

FUNCTIONAL AND MECHANISTIC STUDY OF DOT1L IN MOUSE EMBRYONIC  
HEMATOPOIESIS

BY

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HEMATOPOIESIS

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## Abstract

DOT1 is the histone 3 lysine 79 methyltransferase with both unique structure and substrate specificity. It plays critical role in telomere silencing maintenance, transcription regulation, DNA repair, and cell cycle regulation. DOT1L in mammals is required for embryonic development. In the absence of DOT1L, mouse embryos die by E13.5. Severe anemia was observed in *Dot1L* mutant embryos at E10.5. In order to study a potential role for DOT1L in embryonic hematopoiesis, colony forming assays were performed using both primitive and definitive yolk sac progenitors. We found that DOT1L is required for both primitive and definitive hematopoietic development in the mouse. Interestingly, there is a specific defect in erythroid lineage development. Using flow cytometric analysis, we showed that *Dot1L* deficiency does not affect the emergence of the progenitor population. When *Dot1L* mutant progenitor cells differentiate into the erythroid lineage, they fail to progress normally through the cell cycle and undergo increased apoptosis when compared to wild type progenitors. In order to investigate the molecular mechanism of DOT1L in hematopoiesis, the expression of genes that are critical in hematopoiesis was analyzed in *Dot1L* mutant progenitors. Although *Gata-2* expression appears to be regulated by DOT1L, the reduction of *Gata-2* in yolk sac progenitors alone does not lead to an observable hematopoietic defect. *Trpc6*, which plays a role in EPO-mediated calcium influx, was down regulated significantly in *Dot1L* mutant progenitors. Based on our data and previous studies demonstrating a role for TRPC proteins in mediating EPO induced calcium influx, I hypothesize that *Dot1L* deficiency results in an abnormal EPO response in hematopoietic progenitor cells. This response is characterized by significantly increased calcium influx induced by EPO. This

abnormal intracellular calcium levels in turn result in the observed defective erythropoiesis. In support of this hypothesis, calcium imaging analysis demonstrated that the intracellular calcium level was much higher in *Dot1L* mutant progenitor cells after EPO treatment in comparison with wild type cells. My study explores the cellular and biological function of *Dot1L* and provides more insight into the complex regulation of embryonic hematopoiesis.

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## Table of Contents

<b>Abstract.....</b>	<b>iii</b>
<b>Acknowledgements .....</b>	<b>v</b>
<b>Table of Contents .....</b>	<b>vii</b>
<b>List of Figures and Tables .....</b>	<b>xi</b>
<b>Abbreviations .....</b>	<b>xiv</b>
<b>Chapter 1 Introduction .....</b>	<b>1</b>
1.1 Embryonic erythropoiesis .....	3
1.1.1 Development of primitive erythropoiesis .....	4
1.1.2 Development of definitive erythropoiesis .....	10
1.1.3 Important regulatory mechanisms of embryonic erythropoiesis .....	14
1.1.3.1 Important transcription factors and coregulators in embryonic erythropoiesis .....	14
1.1.3.2 The important function of EPO during erythropoiesis .....	22
1.2 Histone H3 lysine 79 (H3K79) methylation by DOT1 methyltransferase .....	28
1.2.1 The characteristics and protein structure of DOT1 .....	29
1.2.2 Important functions of DOT1 .....	34
<b>Chapter 2 Materials and Methods .....</b>	<b>44</b>
2.1 Standard solutions and reagents .....	44
2.2 Animal Husbandry .....	47
2.2.1 Generation of Dot1L mutant mice and breeding scheme .....	47
2.2.2 Genotyping of progeny .....	47
2.2.3 Animal euthanasia .....	47

2.3 DNA manipulations .....	48
2.3.1 Genomic DNA isolation .....	48
2.3.2 Polymerase Chain Reaction (PCR) .....	48
2.4 RNA manipulations .....	49
2.4.1 RNA isolation .....	49
2.4.2 Reverse Transcriptase (RT)-PCR .....	50
2.4.3 Real-time PCR for gene expression analysis .....	50
2.5 Preparation of single yolk sac cell suspension .....	50
2.6 Culture of mouse embryonic fibroblasts .....	51
2.7 Extraction of histone from both starved and restimulated MEF .....	51
2.8 Whole-mount immunolabeling for fluorescence .....	51
2.9 Analysis of primitive and definitive hematopoiesis by	
colony forming unit assay .....	52
2.10 Benzidine staining of erythroid colonies or	
hemoglobin-containing K562 cells .....	53
2.11 Giemsa staining of blood smear of circulating	
blood from E10.5 embryos .....	53
2.12 Cell cycle and apoptotic study .....	54
2.13 K562 cell hemin induction .....	55
2.14 <i>DotIL</i> knock down in K562 cells .....	55
2.15 Lentiviral transduction of G1E cells .....	56
2.16 Bone marrow and liver cell extraction from tissue .....	57
2.17 Western Blot analyses .....	58



2.18 Chromatin immunoprecipitation (ChIP) assay .....	59
2.19 Flow cytometric analysis and fluorescence activated cell sorting .....	63
2.20 Measure calcium concentration changes in yolk sac cells in response to EPO .....	63

### **Chapter 3 Early mammalian erythropoiesis requires the DOT1L**

<b>Methyltransferase .....</b>	<b>65</b>
3.1 Introduction .....	65
3.2 Results .....	67
3.2.1 <i>Generation of mouse lines containing mutant allele of Dot1L</i> .....	67
3.2.2 <i>Dot1L deficiency results in embryonic lethality and severe anemia</i> .....	71
3.2.3 <i>DOT1L controls yolk sac erythropoiesis</i> .....	73
3.2.4 <i>Dot1L deficiency blocks cell-cycle progression and promotes apoptosis of hematopoietic progenitor cells</i> .....	82
3.3 Discussion .....	84

### **Chapter 4 Mechanistic studies of DOT1L in embryonic erythropoiesis ..... 90**

4.1 Introduction .....	90
4.2 Results .....	93
4.2.1 <i>Expression patterns of certain genes that are critical in hematopoiesis are changed in Dot1L mutant progenitor cells</i> .....	93
4.2.2 <i>Gata-2 expression is regulated by DOT1L, but this is not the mechanism by which DOT1L regulates hematopoiesis</i> .....	101
4.2.3 <i>Trpc6 expression is affected in Dot1L deficient yolk sac progenitor cells and this may contribute to the erythropoietic defect</i> .....	108

4.3 Discussion .....	118
<b>Chapter 5 General Discussions .....</b>	<b>124</b>
5.1 The diverse functions of DOT1 .....	125
5.2 Mechanistic studies of <i>Dot1L</i> in hematopoiesis .....	130
5.3 Future directions .....	133
5.4 Conclusion remarks .....	136
<b>References .....</b>	<b>137</b>
<b>Appendix .....</b>	<b>155</b>

## List of Figures and Tables

Figure 1.1 The step-wise progression of hematopoietic development in mouse embryo .....	2
Figure 1.2 Different models of yolk sac blood islands .....	6
Figure 1.3 Morphological comparison between primitive and definitive erythroid colonies and erythrocytes .....	7
Figure 1.4 The location of H3K79 on the nucleosome .....	32
Figure 1.5 Illustration of gene trap .....	41
Figure 3.1. Generation of mice with mutant <i>Dot1L</i> alleles .....	69
Figure 3.2 <i>Dot1L</i> expression levels in WT, heterozygous, and <i>Dot1L</i> KO cells .....	70
Figure 3.3 Defects in hematopoiesis and vascular development in <i>Dot1L</i> KO embryos .....	72
Figure 3.4 Blood smears of circulating blood from E10.5 embryos .....	74
Figure 3.5 Loss of DOT1L results in the impairment of both primitive and definitive yolk sac erythropoiesis .....	75
Figure 3.6 Benzidine staining of primitive and definitive erythroid colonies .....	77
Figure 3.7 Giemsa staining of cells from WT and KO primitive and definitive erythroid colonies .....	79
Figure 3.8 C-kit/CD41 staining of E10.5 yolk sacs from WT and <i>Dot1L</i> KO embryos .....	80
Figure 3.9 c-kit <sup>+</sup> /CD45 <sup>+</sup> cells in E10.5 yolk sacs .....	81
Figure 3.10 Defects in cell cycle progression and increased apoptosis caused by <i>Dot1L</i> knockout during erythropoiesis .....	83

Figure 4.1 Calcium influx in response to EPO .....	92
Figure 4.2. Gene expression analysis of wildtype and <i>Dot1L</i> knockout progenitor cells in yolk sac .....	94
Figure 4.3 <i>Gata-2</i> and <i>pu.1</i> expression in G1E cells .....	96
Figure 4.4. H3K79 methylation in the <i>Gata-2</i> and <i>Pu1/Sfi1</i> loci .....	97
Figure 4.5 <i>Trpc6</i> expression in bone marrow and liver .....	99
Figure 4.6 H3K79 methylation in <i>Trpc6</i> locus .....	100
Figure 4.7 <i>Dot1</i> knock down with pGIPz vector changes the expression level of <i>Gata-2</i> .....	103
Figure 4.8 <i>Dot1</i> knock down with pLKO.1 vector .....	105
Figure 4.9 <i>Gata-2</i> +/- yolk sac progenitors did not show hematopoietic defect in CFU Assay .....	107
Figure 4.10 Hemin induction on K562 cells .....	109
Figure 4.11 <i>Dot1L</i> deficiency results in TRPC6 protein level reduction in c-kit+ yolk sac cells .....	112
Figure 4.12 Intracellular calcium change in yolk sac cells upon EPO treatment .....	114
Figure 4.13 Erythropoietic defect of <i>Dot1L</i> progenitor cells can not be rescued by adding EGTA to growth medium .....	117

Table 1.1 Comparison between primitive and definitive erythropoiesis	
during mouse embryonic development .....	13
Table 3.1 Survival rate at different stages .....	70

## Abbreviations

AGM	aorta-gonad-mesonephros region
BSA	bovine serum albumin
bp	base pair
BFU-E	erythroid burst-forming unit
CFU-E	erythroid colony-forming unit
CFU-GM	granulocyte macrophage colony-forming unit
ChIP	chromatin immunoprecipitation
°C	degrees centigrade
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOT1	disruptor of telomeric silencing
DOT1L	DOT1-like
E	embryonic (day)
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EryP-CFC	primitive erythroid colony-forming cells
ES	Embryonic stem (cells)
EtOH	ethanol
EPO	erythropoietin

FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GFP	green fluorescent protein
HSC	hematopoietic stem cell
IACUC	Institutional Animal Care and Use Committee
IMDM	Iscove's Modified Dulbecco's Media
iPS cell	induced pluripotent stem cell
kb	kilo base
KO	knock out
KD	knock down
L	liter
M	molar
MEFs	mouse embryonic fibroblasts
mg	milligram
min	minutes
ml	milliliter
MLL	mixed lineage leukemia
mM	millimolar
mm	millimeter
ng	nanogram
PCR	polymerase chain reaction

PBS	phosphate buffered saline
PI3K	phosphatidylinositol-3-kinase
PLC $\gamma$	phospholipase C gamma
RA	retinoic acid
RNA	ribonucleic acid
rpm	rounds per minute
RT-PCR	reverse transcriptase PCR
SCF	stem cell factor
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
sec	second
SOP	standard operating procedure
vol	volume
WT	wild type
wt	weight
x g	times gravity
$\mu$ g	micro gram
$\mu$ l	micro liter
$\mu$ M	micro molar
$\mu$ m	micro meter

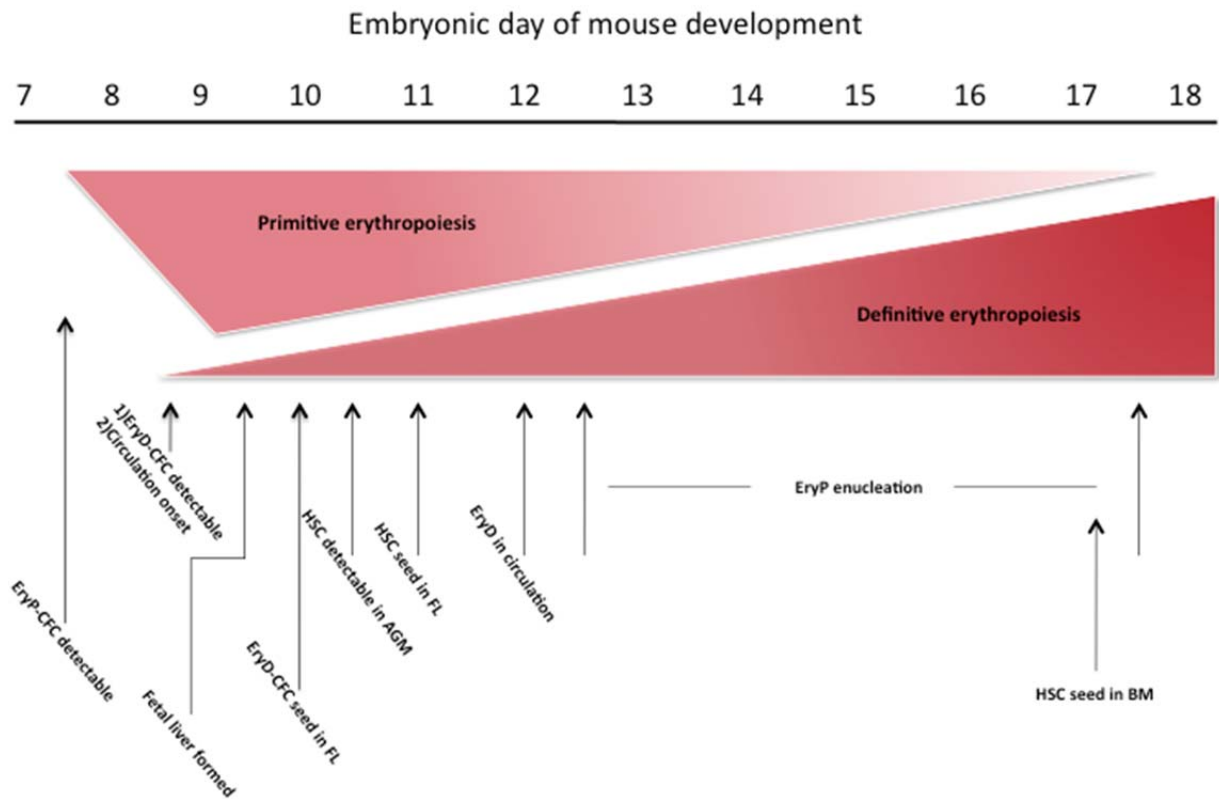


# Chapter 1

## Introduction

Red blood cells are not only critical for the well-being of adults, but are also essential for survival and development of the mammalian embryo beyond the early postimplantation stages<sup>1</sup>. The embryo's first wave of "primitive" erythrocytes are derived from a transient group of committed progenitors from mesoderm-derived cells and are first found in the yolk sac as immature precursors<sup>2</sup>. They then enter the blood stream and differentiate in a semisynchronous manner. This first wave of red cell maturation, known as "primitive" erythropoiesis, is accompanied by globin gene switching and enucleation. Before the end of this first wave of blood development is finished, a second, "definitive" wave of erythropoiesis is initiated in the yolk sac<sup>3</sup>. These definitive erythroid progenitors enter the blood stream and seed the fetal liver, where mature erythrocytes are released into blood stream. At nearly the same time, hematopoietic stem cells (HSC), which are believed to be the source of long-term erythroid potential, emerge in the embryo and also seed the liver. Toward the end of gestation, definitive erythropoiesis shifts from the fetal liver to its final site, the bone marrow<sup>1</sup> (Figure 1.1).

Erythropoiesis is a complicated process, with multiple levels of strict regulation at various points throughout the process. For instance, at the multipotential progenitor stage, the ratio of key transcription factors that are important for lineage determination must be controlled precisely, so that the development of each lineage can be ensured<sup>4,5</sup>. Also, erythroid cells must respond properly to certain cytokines, such as EPO, at certain stages of development, so they can proliferate and differentiate normally<sup>4,6</sup>. Thus, expression of cytokines, their receptors, as well as the appropriate factors comprising the specific



**Figure 1.1 The step-wise progression of hematopoietic development in mouse**

**embryo.** There are two waves of erythroid progenitors emerging in the mouse embryo.

The first wave consists of primitive erythroid progenitors (EryP-CFC) that emerge in the yolk sac at about E7.5. They generate primitive erythroid precursors and start the process of enucleation from E12.5. The second wave consists of definitive erythroid progenitors (EryD-CFC) and long term hematopoietic stem cells (HSC) from different locations, including yolk sac, aorta-gonad-mesonephros (AGM), placenta, fetal liver (FL), bone marrow (BM). Definitive progenitors and HSC use fetal liver and then bone marrow as their maturation site.

signaling apparatus associated with that factor must be expressed or down-regulated at the appropriate times and at appropriate levels. Among factors that contribute to the regulation and maintenance of erythropoiesis, the histone 3 lysine 79 methylation has emerged as an important player<sup>7</sup>. The enzyme that catalyzes this modification, Disruptor of Telomere silencing (DOT) 1, is expressed in different species and its functions have been studied extensively by many groups<sup>8-14</sup>. Our recent study has shown that DOT1L (DOT1 Like, the DOT1 mammalian ortholog) is required for mouse embryonic development, particularly playing a role in erythropoiesis<sup>7</sup>. Recently it has been shown by other groups that DOT1L is also required for adult hematopoiesis<sup>15</sup>. However, the molecular mechanism(s) by which DOT1L regulates erythropoiesis is not completely understood. In this chapter, I summarize our current understanding about embryonic erythropoiesis, DOT1L structure and functions, and recent progress in DOT1L studies.

## 1.1 Embryonic Erythropoiesis

Developmentally, erythropoiesis in the mouse begins in the yolk sac/blood islands, later at the aorta-gonad mesonephros (AGM) region, the placenta, the fetal liver, and at last in the bone marrow toward the end of gestation<sup>1,16</sup>. In addition to the important function of delivering oxygen, red cells in the embryo encounter at least two great challenges compared to their adult counterparts. First, the fast-developing embryo at postimplantation stages requires functional red cells before the long-term hematopoietic stem cell (HSC) and their microenvironmental niches are established. Second, since the mammalian embryo grows very fast, embryonic erythropoiesis must generate ever-increasing numbers of red cells to meet the needs of the growth of the embryo. It has been estimated that from embryonic day 12.5 (E12.5) to E16.5, an increase of 70-fold in

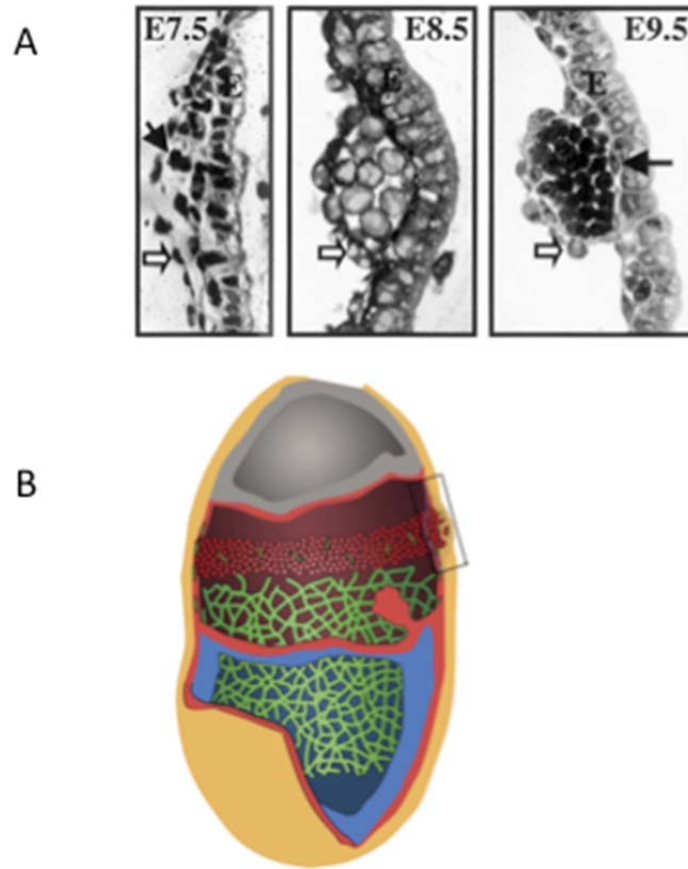
red cell mass has to be achieved<sup>1</sup>. Due to its critical function and unique properties, embryonic erythropoiesis has been studied for very long time. Nearly 100 years ago, it was already recognized that two distinct populations of red cells circulate in the blood stream of mouse embryos. The first population, the “primitive” red cells, are extremely large, nucleated red cells, which originate from the yolk sac<sup>2</sup>. Shortly after that, a second, “definitive” population, consisting of smaller, enucleated red cells supersedes the primitive cells and continues to circulate during the fetal stage and for the entire life of the organism<sup>3</sup>. The paradigm of embryonic erythropoiesis has changed immensely due to our increasing knowledge over time. For example, at first the primitive red cells are thought to be always nucleated and have the same characteristics with the nucleated nonmammalian red cells. Also originally, people believed that definitive erythroid cells were derived from a single location. Recent findings have challenged all of these long-held opinions. In this section, I will summarize our current understanding of embryonic erythropoietic development, as well as some of the important regulatory mechanisms that regulate this stage.

#### 1.1.1 Development of primitive erythropoiesis

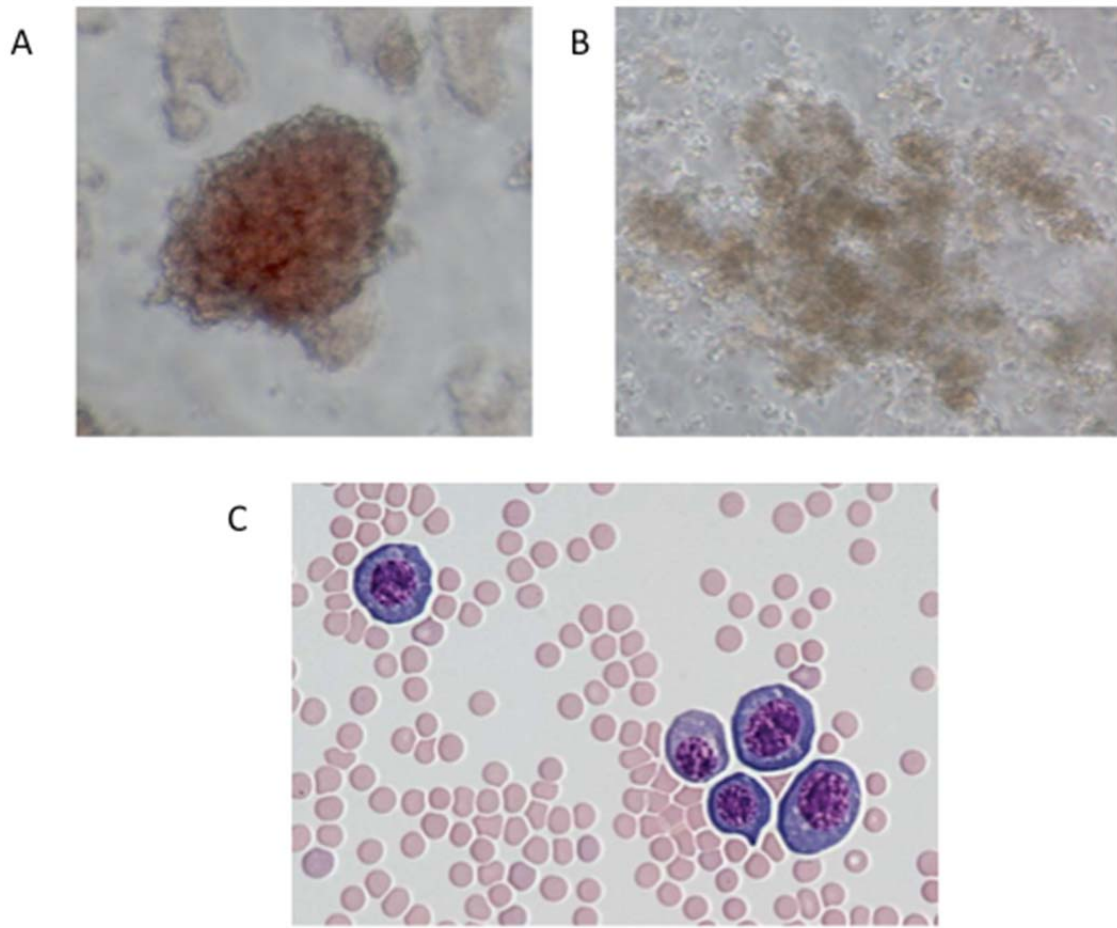
During gastrulation, mesoderm cells emerge from primitive streak, which is a posterior midline structure of the epiblast. The initial generation of primitive erythrocytes relies on the normal development of mesoderm cells. They then migrate to the extraembryonic yolk sac, where they differentiate into primitive erythropoietic cells and endothelial cells<sup>17</sup>. It has long been recognized that the yolk sac blood island is the first site of hematopoiesis and origin of primitive erythrocytes. The yolk sac is a bilayered structure, composed of inner mesoderm and outer visceral endoderm. Blood islands are

isolated structures consisting of a cluster of primitive erythroblasts surrounded by these two layers<sup>18</sup> (Figure 1.2 A). This is the traditional definition of blood islands. Although the importance of blood islands as the origin of primitive erythropoiesis has been commonly recognized, there is still debate about their morphology. A global view of early yolk sac blood and endothelial cell development has been achieved thanks to the application of whole mount *in situ* hybridization and fluorescent immunohistochemistry on the whole embryos. It showed that in the yolk sac at E7.75, the blood cells form a complete circumferential band in the blood island region. A cross-sectional image of this band will give people the impression of isolated island. The band of blood cells is subsequently enveloped and then divided by endothelial cells to form many blood-filled endothelial channels. When cut cross-sectionally, a single channel would also give people the impression of an “isolated” island, which in fact is just an artifact of the sectioning and is not a complete view<sup>18</sup> (Figure 1.2 B).

Primitive erythroid progenitors can be identified in the embryo shortly after gastrulation as early as E7.25. Recent studies have shown that the expression of CD41 (integrin  $\alpha$  IIb) is an accurate marker for the earliest erythropoiesis in the yolk sac of developing embryo<sup>19-21</sup>. The primitive erythroblasts are the descendants of these unique CD41-positive progenitors, called primitive erythroid colony-forming cells (EryP-CFC)<sup>22</sup>. The colonies derived from these progenitors are distinct from definitive erythroid colonies in at least three aspects: first, their maturation time is relatively short (5 days); second, their globin gene expression pattern is unique; third, the morphology of the colony as well as the individual red cells within the colony is different<sup>1</sup> (Figure 1.3).



**Figure 1.2 Different models of yolk sac blood islands.** (A). Traditional model of blood island development in the mouse embryo between E7.5 and E9.5. Yolk sac blood islands form between the single cell layer of endoderm cells (E) and mesothelial cells (open arrow). Blood islands develop from undifferentiated mesoderm cells (E7.5, closed arrow) that give rise to inner blood cells surrounded by an outer endothelial lining (E9.5, closed arrow). (B) A novel model of a “blood island” as a complete band of primitive erythroid progenitors (red) and sparse endothelial cells (green) in the blood island region of the yolk sac. The primitive capillary plexus (green mesh) separates the blood band from the distal embryo proper. Inset box indicates how the blood band would appear in section as shown in traditional model. Panel A is from Palis et al, 2001<sup>23</sup>. Panel B is from Ferkowicz et al, 2005<sup>18</sup>.



**Figure 1.3 Morphological comparison between primitive and definitive erythroid colonies and erythrocytes.** (A-B) Representative pictures of primitive (A) and definitive (B) erythroid colonies. (C) Circulating blood cells were isolated both from E9.5 embryos and from adult mice, mixed together, cytopun, and stained with Wright-Giemsa. Primitive erythrocytes are nucleated and significant larger than the definitive erythrocytes. Panel C is from McGrath et al, 2008<sup>1</sup>.

After EryP-CFC are identified in the embryo at about E7.25, they expand very fast and reach the peak in numbers at E8.0. After that, their numbers rapidly decrease, and they can no longer be found after E9.0<sup>24</sup>. Although the nature of EryP-CFC is transient, they are critical for embryo survival and development. The progeny cells of the primitive progenitors remain in the circulation of embryo for a prolonged time, together with definitive red cells released from fetal liver later on (E12) to perform their function of providing sufficient oxygen to the embryo until a relatively late stage of gestation<sup>1,24</sup>. Therefore it is easy to understand that anemia observed before E13 in mouse must be attributed to loss or decreased number of primitive red cells.

Primitive erythroblasts are released into circulation while they are still immature, and it is believed that they undergo maturation in the blood stream<sup>1,25</sup>. There are two important aspects for erythroid maturation: enucleation and hemoglobin accumulation. It has been believed for long time that primitive red cells remain nucleated state for all their lifetime, a characteristic shared with nucleated red cells of nonmammalian vertebrates. However, recent studies have challenged this long-held opinion by showing that primitive erythroblasts in the mouse embryo ultimately enucleate, just like their definitive counterpart. The time range for mouse primitive erythroblast enucleation is between E12.5 and E17.5<sup>26</sup>. Unlike definitive erythroblasts, which mature in erythroblast islands of the fetal liver and postnatal bone marrow, primitive erythroblasts are actively circulating in the blood stream. Therefore the exact location and mechanism for their enucleation is still elusive.

A recent study using transgenic mouse models has provided more insight into the development of primitive erythroid cells<sup>27</sup>. In this study, human epsilon-globin gene



regulatory elements have been used to drive GFP expression. Only primitive erythroid cells express GFP. This is a better and more accurate way to trace primitive erythroid cells than by flow cytometry (after staining the cells with lineage markers, such as CD41 or Tie2). Three developmental niches for primitive progenitor cells were identified in this study: yolk sac, blood stream, and fetal liver. The accumulation of strong GFP-expressing primitive erythroblasts in the fetal liver was their new finding, since most people assumed that fetal liver was the site of maturation for definitive erythroblasts. What they found was that erythroblasts accumulate transiently within fetal liver and often within erythroblastic islands, in close contact with fetal liver macrophages. Importantly, they found that nuclei of primitive erythroblasts labeled by expression of histone-H2B-EGFP were within fetal liver macrophages in both *in vitro* culture or *in vivo* conditions, suggesting a possible mechanism whereby primitive erythroblast nuclei were cleared and degraded by macrophages at least in fetal liver.

The second important aspect of primitive erythroblast maturation is the accumulation of hemoglobin. They accumulate increasing amounts of hemoglobin and reach their steady-state hemoglobin content (80-100 pg per cell), which is about six times the amount of hemoglobin found in definitive erythrocytes. This is in accordance with the fact that primitive erythroblasts are about six times larger than definitive erythroblasts<sup>1</sup>. It has been recognized for several decades that primitive erythroblasts contained different hemoglobins when compared with their adult counterpart<sup>28</sup>. The primitive red cells in mouse express not only embryonic globins (such as  $\beta$  H1 and  $\epsilon$  y) but also express low levels of adult type globins ( $\beta$  1 and  $\beta$  2). Embryonic hemoglobins in the mouse have relatively high affinity to oxygen compared to adult hemoglobins, which potentially

facilitates oxygen exchange across the placenta. Interestingly, as primitive erythroblasts terminally differentiate, they undergo a process termed “maturational globin switching” in which the initially expressed  $\zeta$  - and  $\beta$  H1-globins are superseded by  $\alpha$  1,  $\alpha$  2 and  $\epsilon$  y-globins<sup>29</sup>. These findings indicate that in the primitive erythroid lineage, the  $\alpha$  and  $\beta$  globin loci are precisely and actively regulated as the cells transition from the progenitor stage to a more mature precursor stage.

#### 1.1.2 Development of definitive erythropoiesis

As I discussed before, since the mammalian embryo grows very fast, embryonic erythropoiesis must generate ever-increasing numbers of red cells to meet the needs of growth. The number of red cells generated by primitive erythropoiesis can only meet the requirements of the early post-implantation stage. In order to fulfill the requirements for cell numbers, a second wave of embryonic erythropoiesis, definitive erythropoiesis, begins 24 hours after the primitive erythropoiesis<sup>1</sup>. This definitive process can give rise to larger numbers of erythrocytes, which co-exist with the primitive red cells persisting throughout gestation.

The site(s) of the origin of definitive progenitors has been a subject of longstanding debate and intense study. Unlike primitive erythropoiesis, which originates from a single site, the yolk sac, definitive erythropoiesis can originate in several possible sites, including the yolk sac (YS), aorta-gonad-mesonephros (AGM), and placenta<sup>1,17</sup>. The earliest definitive erythropoiesis potential can be identified in the yolk sac at about E8.5 in the mouse. The progenitor cells, however, do not become mature in the yolk sac. At this time, the fetal liver serves as the site of the maturation of definitive erythroid cells. Definitive progenitors enter the blood stream and seed the fetal liver as soon as it

begins to form as an organ at E9.5<sup>1</sup>. The source of the yolk sac progenitors has not been clearly resolved. Some groups have suggested that progenitor cells with definitive potential from the aorta region may migrate and take up in the yolk sac niche and undergo erythropoiesis. While theoretically this may happen, there is currently no strong evidence for this. Recently, a study using the *Ncx1* KO mouse showed that all primitive and definitive hematopoietic progenitor cells before E10 in the mouse embryo are derived from the yolk sac<sup>17</sup>. *Ncx1* KO embryonic mice die at E11 because they do not have cardiac contractions, but hematopoiesis from E7.0 to E9.5 remains intact. In this study, they showed that the total number of primitive and definitive progenitors between *Ncx1* KO and WT is quite similar, but in *Ncx1* KO embryos, neither primitive erythroblasts nor definitive hematopoietic progenitors could be found in the para-aortic splanchnopleura (PSP, a precursor to the AGM) from E8.25 to E9.5. This study indicated that all the primitive red cells in the circulation and the definitive progenitors that seed the liver before E10 are derived from the yolk sac.

Although definitive erythrocytes derived from yolk sac meet the needs of embryonic growth temporarily, the continuous generation of blood cells from long-term hematopoietic stem cells (HSC) is the ultimate goal of adult hematopoiesis. The first long-term HSC, that have the capacity of engrafting adult recipients, have been identified in the AGM region at E10.5<sup>30</sup>. In addition to the AGM region, the placenta is also a possible origin of HSC and can serve as a site of HSC proliferation<sup>31</sup>. HSC then migrate through the blood stream and seed the fetal liver at E11. The numbers of definitive hematopoietic progenitors (BFU-E and CFU-E) increase exponentially for several days in the fetal liver and reach a peak at around E14.5-E15.5. Subsequently, the hematopoietic

niche shifts gradually from fetal liver to the bone marrow cavity and the fetal liver stops functioning as an hematopoietic organ soon after birth<sup>1</sup>. There is still a disagreement in defining definitive erythropoiesis. Some people believe that it should be further divided into two waves: one process in which erythrocytes are derived from yolk sac progenitors and another in which erythrocytes are derived from long-term HSC. The principal differences between the two reside in the cells' different origins and their slightly distinct morphologies<sup>1</sup>. Other researchers simply group the two populations together and consider them both as having the potential to undergo definitive erythropoiesis<sup>24</sup>. I prefer the latter opinion since it is more concise, and use this as a model of embryonic erythropoiesis (see Figure 1.1).

The origin(s) of primitive and definitive erythroid progenitors has been an interesting topic for very long time. Since erythroid and endothelial cells have very close spatial and temporal relationship and share some important transcription factors and markers, the term “hemangioblast” was coined 100 years ago<sup>32</sup>. These common endothelial and hematopoietic ancestors first appear in the primitive streak in the embryo at E7.5. Then the endothelial cells with hematopoietic potential can be identified lining the blood islands of yolk sac. The development of *in vitro* model of embryonic hematopoiesis was crucial in the eventual identification of the hemangioblast. The *in vitro* equivalent of the hemangioblast is termed blast colony forming cell (BL-CFC), which can generate a blast colony containing both hematopoietic and endothelial cells<sup>33</sup>. The BL-CFC expresses both fetal liver kinase 1 (Flk-1), the vascular endothelial growth factor receptor 2, and the mesodermal marker Brachyury. Some researchers have challenged the existence of hemangioblasts. For example, Ueno et al. claimed that

hematopoietic cells are coming mostly from Flk-1<sup>-</sup> cells while the endothelial cells are generated from Flk-1<sup>+</sup> cells<sup>34</sup>. Soon after that, however, another group refuted their results using a simpler and more efficient lineage tracing system, showing that most primitive and definitive blood cells are the progeny of Flk-1 expressing hemangioblasts<sup>35</sup>. Another group also demonstrated that hematopoietic cells are generated from hemangioblasts through a hemogenic endothelium stage, which is the intermediate linking hemangioblast and hematopoietic progenitor<sup>36</sup>. With accumulated data, more and more researchers tend to believe in the existence of hemangioblasts. Table 1 is a summary of the important properties of both primitive and definitive erythropoiesis.

Table 1.1 Comparison between primitive and definitive erythropoiesis during mouse embryonic development

	<b>Primitive</b>	<b>Definitive</b>
Progenitors	<i>EryP-CFC</i>	BFU-E, CFU-E
Emerging time	E7.25	E8.5
Colony formation time	5 days	BFU-E 7-10 days, CFU-E 2-3 days
Site of maturation	Blood stream, fetal liver	Fetal liver, bone marrow
Cell size	Larger (about 6 times that of definitive)	smaller
Hemoglobin accumulation	80-100pg	12-20 pg
$\alpha$ globin expression	$\zeta$ , $\alpha 1$ , $\alpha 2$	$\alpha 1$ , $\alpha 2$
$\beta$ globin expression	$\beta H1$ , $\epsilon \gamma$ , $\beta 1$ , $\beta 2$	$\beta 1$ , $\beta 2$

### 1.1.3 Important regulatory mechanisms of embryonic erythropoiesis.

Embryonic erythropoiesis in the mouse is a dynamic and complicated process that requires the coordinated actions of many molecular mechanisms. When oxygen tension in the developing embryo arises, larger numbers of erythrocytes is required to be generated to meet the need. This is achieved by increasing red cell proliferation, reducing apoptosis, and promoting red cell maturation, through a complex network of interactions of transcription factors, responses to certain growth factors, and the activation of important signal transduction pathways<sup>6</sup>. Since the regulation of embryonic erythropoiesis is such a broad and complex topic and will be very hard to cover within a single discussion, only two important aspects of embryonic erythropoiesis which are involved directly in my research will be discussed in the introduction: first, important transcription factors and coregulators; second, the important function of erythropoietin (EPO).

#### 1.1.3.1 Important transcription factors and coregulators in embryonic erythropoiesis.

When erythroid progenitor cells encounter specific stimuli, such as low oxygen tension or cytokines, such as SCF, an erythroid-specific transcription program will be created, through which the progenitor cells will fulfill differentiation along different lineages<sup>37</sup>. Erythroid transcription factors were first identified through their binding to the promoter regions of some erythroid-specific genes, such as the  $\beta$ -globin gene. Their critical functions have been confirmed by both gene knockouts in *in vivo* systems and transcription factor null hematopoietic cell lines, *in vitro*. In addition, the precise gene target networks on a global scale as well as the cis-acting elements of some of the

important transcription factors have been identified by chromatin immunoprecipitation (ChIP). Through these studies, it has been found that some transcription factors have dual functions. They not only activate gene expression required for the generation of the erythroid lineage, but they also exert suppression of gene expression of factors important for the development of alternative hematopoietic lineages. The important features of several key transcription factors in embryonic erythropoiesis and the interactions between some of them are discussed below.

**GATA-1.** GATA-1 is the founding member of a small family of zinc finger transcription factors that recognize the DNA motif (T/A(GATA)A/G). It was originally isolated as a binding protein to the promoter region of the  $\beta$ -globin gene. Subsequent studies showed that it is expressed broadly in the erythroid, megakaryocyte, eosinophil, and mast cell lineages<sup>38</sup>. There are three functional domains on the GATA-1 protein. The N-terminal domain acts as transcriptional activation domain. Two zinc finger domains are located toward the C-terminus of the protein. The zinc finger close to the C-terminus recognizes the GATA motifs. The zinc finger close to N-terminus helps stabilize DNA binding and has the capacity to interact with other protein factors, such as FOG-1, and erythroid Kruppel-like factor (EKLF)<sup>39</sup>.

GATA-1 plays very important role in embryonic erythropoiesis. Loss of GATA-1 results in embryonic lethality at around E10.5-E11.5 due to severe anemia<sup>40</sup>. GATA-1 can promote erythropoiesis by activating transcription of genes that are associated with mature red blood cell function and phenotype. GATA-1 null cells (both primitive and definitive erythroid progenitors) are not able to differentiate beyond the proerythroblast stage and undergo apoptosis<sup>41</sup>. GATA-1 also performs its function by repressing gene

expression. For example, GATA-1 can inhibit the transcription of c-kit and GATA-2, whose expression are high at a relatively early stage of embryonic erythropoiesis. The c-kit and GATA-2 loci contain GATA motifs and are bound by GATA-2 factor at early stage of erythroid progenitors, which promotes the transcription of c-kit and GATA-2. The expression of GATA-2 and c-kit at this stage is critical for early progenitor survival and proliferation. During relatively late stages, GATA-1 expression predominates and replaces GATA-2 at the same sites to inhibit GATA-2 and c-kit transcription. By doing this, it can help cells in the erythroid lineage undergo maturation<sup>4</sup>.

**FOG-1** stands for Friend of GATA. As the name implies, it is a very close interacting partner of GATA-1, identified through a yeast two-hybrid screen. It is a large protein factor containing nine zinc fingers<sup>42</sup>. FOG-1 is expressed in erythroid cells as well as megakaryocytic cells. Its expression pattern is identical to that of GATA-1 and the phenotype of the FOG-1 knockout is quite similar to that of the GATA-1 knockout. Both result in embryonic lethality at nearly the same stage due to anemia<sup>43</sup>. The close relationship between FOG-1 and GATA-1 is further highlighted by an experiment in which the interaction of these two proteins was disrupted by a point mutation. By doing this, the experiment showed strong evidence that GATA-1/FOG-1 interaction was essential for erythroid differentiation. This study also showed that this protein interaction promoted erythroid differentiation by activation and repression of GATA-1 target genes<sup>44</sup>. Despite all these findings, the molecular mechanisms underlying FOG-1 dependent transcriptional activation and repression are poorly understood. One possibility is that FOG-1 facilitates GATA-1 chromatin occupancy at certain loci. Another



possibility is that FOG-1 helps facilitate GATA-1-mediated DNA looping, which juxtapose the distal enhancer elements to the promoter region, as seen in  $\beta$ -globin locus<sup>4</sup>.

**GATA-2** is another important member of the GATA family of zinc finger transcription factors. It is expressed in hematopoietic stem cells and progenitor cells, endothelial cells, and some other tissues, including the central nervous system, placenta, fetal liver and heart. GATA-2 knockout experiments in mice showed that no GATA-2 knockout embryos survived beyond E11.5. At E10.5, most of the GATA-2 knockout embryos (more than 60%) were dead due to anemia, as demonstrated by pale embryos and nearly empty yolk sac vessels<sup>45,46</sup>. In the *in vivo* and *in vitro* differentiation analyses using GATA-2 null embryonic stem cells, GATA-2 was shown to be required for survival and proliferation of immature erythroid progenitors<sup>47</sup>. Overexpression of GATA-2, induced proliferation of progenitors and inhibited progenitor cell maturation<sup>48</sup>.

Despite the unique expression patterns and developmental functions of GATA-1 and GATA-2, there is considerable interplay between these two factors. As we discussed before, GATA-1 can inhibit GATA-2 function and decrease the expression levels of GATA-2 target genes at a relatively late stage. This is the so-called “GATA switch” in which GATA-2 is displaced from its binding sites by rising GATA-1 levels, facilitated by FOG-1<sup>5</sup>. This is a very important process in that it demonstrates the dynamics of transcriptional regulation during embryonic erythropoiesis, through which different transcription programs are created to meet specific developmental requirements.

In addition to having opposing effects, recent studies have also showed that GATA-2 and GATA-1 can work cooperatively and successively to exert repressive effects on gene expression (such as PU.1) during hematopoietic development<sup>49</sup>.

**PU.1.** PU.1 is encoded by the *sfpi1* gene located on chromosome 2 in the mouse. The Ets domain on the PU.1 protein corresponds to the DNA binding domain, and can recognize the GGAA motif. This Ets domain is also responsible for protein-protein interactions between PU.1 and other factors, such as GATA-1 and Runx1. A C-terminal acidic domain and a glutamine-rich domain encode the transcriptional activation activities<sup>37,50</sup>.

PU.1 expression is mostly restricted to the hematopoietic system and its expression pattern is highly dynamic, characterized by sharp on/off transitions<sup>50</sup>. For example, PU.1 is actively expressed in early hematopoietic cells, including hematopoietic stem cells and multipotential progenitor cells. However, once the progenitor cells become committed to the erythroid/megakaryotic lineage, a marked decrease in PU.1 expression is observed. In erythroid cells, PU.1 expression is low in immature stages (CD71+TER119- population), and becomes completely undetectable in more mature cells (CD71+TER119+)<sup>50,51</sup>. It is believed that during embryonic erythropoiesis, a low level of PU.1 is required for promoting the self-renewal potential of erythroid progenitors. PU.1 knockout embryos die in late gestation and are devoid of fetal liver B lymphocytes, granulocytes, and macrophages. PU.1 null progenitor cells showed reduced proliferative capacity<sup>52</sup>.

Although so far, most data support an important role of PU.1 in the development of the myeloid lineage, it is also very important to note that interactions between PU.1 and GATA family proteins are critical for the development of erythropoiesis. People have found that PU.1, GATA-1 and GATA-2 are all expressed in multipotential progenitor cells<sup>49</sup>. During lineage commitment, the activation of specific transcription factors and the interactions between different factors will direct the

progenitor cells down a particular differentiation pathway. The interaction between these two protein families has been demonstrated by several studies in the past ten years. First, GATA proteins inhibit the functions of PU.1. GATA-2 and GATA-1 can work together and successively to inhibit PU.1 function in the erythroid lineage<sup>49</sup>. Specifically, PU.1 needs to perform its full transcriptional activity by binding to its cofactor c-Jun. However GATA proteins can interact with a small region no larger than 12 amino acids within the PU.1 C-terminal Ets domain. As a result of this binding, GATA proteins could destroy the interaction between PU.1 and c-Jun<sup>53</sup>. It has also been showed that GATA-1 inhibits PU.1 not only by physical protein interaction, but also by repressing PU.1 transcription<sup>49</sup>. On the other hand, PU.1 can inhibit GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding<sup>54</sup>. Interestingly, PU.1 inhibits GATA-1 function not through the same C-terminal Ets domain. In contrast, it has been found that the N-terminal 70 amino acids of PU.1 are essential to repress GATA-1 function by blocking its DNA binding. PU.1 can also inhibit GATA-2 expression<sup>51</sup>. Therefore, we can see that the relative amount of GATA proteins and PU.1 and their interactions are critical in determining lineage commitment of progenitor cells. A negative cross-talk between PU.1 and GATA proteins has been established as a model to describe their functions and interactions. In stem cells or multipotential progenitor cells, GATA-2 predominates to help maintain the expression of PU.1 at an intermediate level. This is consistent with the concept “lineage priming” whereby both myeloid genes (PU.1) and erythroid genes (GATA-2) are expressed at moderate, but detectible levels in multipotential progenitors, to help maintain their plasticity. Upon erythroid/megakaryocytic differentiation, GATA-1 is highly expressed and displaces GATA-2 through the so called “GATA switch”.

GATA-1 is thought to be a stronger repressor of PU.1 and it can repress PU.1 at both the transcriptional and protein levels. Therefore PU.1 function is shut down completely. At the same time, GATA-1 helps ensure terminal erythrocyte maturation. Alternatively, during myeloid differentiation, both GATA-1 and GATA-2 are repressed, which allows for higher-level PU.1 expression and interaction of PU.1 with c-Jun, and consequently the increased expression of downstream target genes<sup>53</sup>.

**EKLF.** The Erythroid Kruppel-like Factor (EKLF) is crucial for erythropoiesis and for  $\beta$  -globin gene expression<sup>5</sup>. It is an erythroid-specific, zinc finger transcription factor and binds to CACC box motifs which can be found in the promoter region of  $\beta$  -globin and many other erythroid genes<sup>55</sup>. The EKLF gene knockout mouse shows embryonic lethality at about E14-15 due to severe anemia<sup>56,57</sup>. When the  $\beta$  -globin gene locus was analyzed in the gene knockout embryos, it showed that the promoter region and the locus control region (LCR) assumed a “closed” chromatin structure, resulting in failed  $\beta$  -globin activation<sup>58</sup>. This indicates that EKLF has an important role in organizing an active chromatin domain in  $\beta$  -globin as well as other erythroid gene loci. Initial studies suggested that EKLF solely plays its role in definitive erythropoiesis. More recent studies demonstrated that it is also involved in primitive erythropoiesis<sup>59</sup>.

**CBP.** The major function of CBP and its paralog p300 is to acetylate histones as well as other non-histone proteins. They are very important in mouse embryonic development. CBP knockout resulted in embryonic lethality due to developmental retardation and defects in multiple systems, including deficient primitive and definitive erythropoiesis<sup>5</sup>. In the hematopoietic system, it plays an essential role in maintaining the self-renewal ability of hematopoietic stem cells and is required for differentiation<sup>60</sup>. CBP can also

expand its capacity by working with other protein factors. For instance, CBP can interact and acetylate GATA-1 and increase the DNA binding ability of GATA-1. Another example is that EKLFs ability to bind a chromatin remodeling complex is greatly enhanced by EKLF acetylation by CBP/p300<sup>5</sup>.

**SCL.** SCL is a member of the basic helix-loop-helix family of transcription factors and binds a DNA motif (CANNTG) called the E-box. SCL performs its function by forming a protein complex, called a “pentameric complex” with other factors, such as LMO2 and GATA-1. The targets of this complex involve many erythroid genes and the regulatory elements of key transcription factors<sup>61</sup>. SCL knockout embryos die before E9.5 and show a complete absence of primitive erythrocytes and myeloid progenitors in yolk sac. SCL null ES cells fail to contribute to any hematopoietic lineage either *in vivo* or *in vitro*<sup>62,63</sup>. Recent studies have shown that SCL is not required for generation of Flk<sup>+</sup> hemangioblasts, but is critical for generation of endothelial and hematopoietic lineages from these cells. After generation of the endothelial lineage and hemogenic endothelium (from which the hematopoietic lineage is derived), SCL is no longer critical, as deletion of SCL in Tie2+, VE-cadherin+ cells does not affect hematopoiesis<sup>64</sup>.

**TRAP220.** TRAP220 or Med1 is the mediator subunit that constitutes a key component of mediator originally found to associate with nuclear hormone receptors<sup>5</sup>. Deletion of TRAP220 results in lethality at E11.5 due to anemia, although the hematopoietic progenitor cell number remains normal. TRAP220 deficient progenitor cells have a defect specifically in erythroid lineage formation (shown by defective BFU-E and CFU-E), but not in the formation of the myeloid lineage<sup>65</sup>. Recent studies using TRAP220 conditional knockout mice, has confirmed this specific block in erythroid development, but not in

myeloid or lymphoid development *in vivo*. Importantly,  $\beta$ -globin gene expression is abrogated in TRAP220 knockout embryos<sup>66</sup>. Therefore, TRAP220 is critical in erythroid development and  $\beta$ -globin gene activation.

#### 1.1.3.2 The important function of EPO during erythropoiesis.

Regulation of erythropoiesis is a complex process involving the function of a number of external factors. Many factors such as cytokines contribute to this process. For example, interleukin-3 has been shown to increase the number of BFU-E, while Stem Cell Factor increases the number of both BFU-E and CFU-E<sup>6</sup>. Receptors for Insulin are expressed at later stages of erythroid differentiation and their ligands show anti-apoptotic effects on differentiating erythroid cells<sup>67</sup>. Transforming Growth Factor  $\alpha$  and  $\beta$  have also been shown to regulate erythrocyte numbers<sup>68</sup>. In addition, some nuclear hormones are also closely involved in erythropoiesis. For instance,  $T_3$  has been shown to regulate proliferation/differentiation of erythropoiesis either positively or negatively at different developmental stages, suggesting that it may exert its function in a time-specific manner<sup>69</sup>.

Among all of these factors, EPO is considered the primary regulator of erythropoiesis. Since the identification of EPO, the life quality of patients with anemia due to chronic kidney disease has been dramatically improved and EPO has become the world's leading biopharmaceutical. The importance of the EPO response is demonstrated by EPO or EPO receptor (EPOR) mutant animals. Mice with mutated EPO/EPOR die due to anemia during embryogenesis at E12.5-13.5, a stage of massive erythroid expansion<sup>70</sup>.

EPO signaling pathway is initiated by EPO's binding to its specific, high-affinity receptors. EPOR was first identified in 1989 and it belongs to the type-I superfamily of

single-transmembrane cytokine receptors. It is expressed in the erythroid progenitor cells derived from bone marrow or yolk sac. It is also found in several non-hematopoietic tissue including myocytes, cortical neurons, and breast and ovarian epithelia<sup>71</sup>. In the erythroid lineage, it is first expressed at the BFU-E stage, and all the way through the orthochromatic erythroblast stage<sup>6</sup>.

When EPO binds EPOR, the receptors will form homo-dimers and undergo a conformational change. The cytoplasmic domain of EPO receptor contains two membrane motifs called Box-1 and Box-2, to which the tyrosine kinase JAK2 binds. The conformational change of EPOR during dimerization induces the reciprocal trans-phosphorylation of JAK2, therefore activating JAK2. Phosphorylated JAK2 can then phosphorylate the eight tyrosine residues on the EPO receptor's cytoplasmic domain, forming a docking site for SH2 containing molecules associated with the EPO signal transduction cascade<sup>6</sup>. From this point, a number of signaling pathways can be initiated:

1) JAK2, in collaboration of a second kinase, Lyn, will activate STAT protein family members. Tyrosine phosphorylation followed by dimerization has been shown to activate ten members of the STAT family, which can act as transcription factors. Among these members, EPO receptors specifically activate STAT5a and STAT5b isoforms. One of the critical target genes of STAT5 is the anti-apoptotic molecule Bcl-X<sub>L</sub>. Anemia was observed in animals with conditional knockout of Bcl-X<sub>L</sub> in the erythroid lineage. Therefore the activation of this pathway by EPO is critical for enhancing survival and proliferation of erythroid progenitors<sup>72</sup>.

2) The EPO response also activates PI3 kinase, which in turn stimulates the PKB/Akt pathway. PKB/Akt pathway has been shown to mediate anti-apoptotic and

proliferative signals. For example, studies using an apoptosis-resistant erythroid cell line showed that activation of PI3 kinase is required to prevent apoptosis. A mouse model with mutated p85  $\alpha$  subunit of PI3 kinase showed markedly reduced BFU-E and CFU-E populations, which resulted in an erythropoiesis defect. Some downstream substrates of PKB/Akt have been proposed. An important example is forkhead box O3A (Foxo3a), a transcription factor that can be inhibited by phosphorylation. The targets of Foxo3a include BTG1 and p27<sup>Kip1</sup>. Both these factors have putative anti-proliferation and anti-differentiation functions<sup>73</sup>.

3) Another pathway activated by EPO is PLC  $\gamma$  signaling. Activation of PLC  $\gamma$  will generate the second messenger inositol-1,4,5-trisphosphate (IP3). Studies have shown that a conformational change of IP3 receptor upon IP3 binding will facilitate its association with the transient receptor potential channel (TRPC) proteins, which are voltage-independent calcium channels<sup>74</sup>. TRPC proteins will then mediate calcium influx into erythroid progenitors, which is required for normal erythropoiesis<sup>75,76</sup>.

Another interesting finding is that during erythropoiesis, EPO does not work alone. A study showed that there was cooperation between c-kit and the EPO receptor, linking these two pathways<sup>77</sup>. SCF and EPO pathways can work at different stages of erythropoiesis to improve cell survival. In the immature erythroid cells, SCF signaling is dominant and induces Bcl-2, EPOR and Stat5 expression. Bcl-2 is the major player of promoting survival. While erythroid cells undergo terminal differentiation, GATA-1 expression results in down-regulation of c-kit and Bcl-2 and up-regulation of EPOR. The increased EPO signaling pathway results in up-regulation of Bcl-X<sub>L</sub>, which replaces Bcl-2 and performs an anti-apoptotic function.



The EPO induced signaling pathways are not limited to the above mentioned. Due to limited space and relevance to my study, only these three are discussed. In the following paragraphs, I am going to discuss the PLC  $\gamma$  and calcium pathways in more detail, because of their strong relevance to our research.

Calcium is a universal signaling molecule that can regulate a variety of aspects of cellular function. New life begins with a surge of calcium at fertilization. Thereafter, this versatile signaling molecule is used again and again in many processes during development and in the adult, including embryonic pattern formation, cell differentiation, cell proliferation, and transcription factor activation<sup>78</sup>. Dysregulation of calcium signaling has been implicated in cancer development<sup>79</sup>. In addition, calcium is one of the key elements in apoptotic signaling pathways. Several calcium-mediated apoptosis mechanisms have been explored. Mitochondria are usually close to the calcium channels of the endoplasmic reticulum (ER). Calcium released from ER will be absorbed by mitochondria, leading to mitochondrial swelling and apoptosis. The calpains, calcium activated cysteine proteases, when induced by some stimuli, can cleave and activate a number of molecules that have important functions in apoptosis, including caspase-12 and Bax. Calmodulin (CaM) is the major sensor of intracellular calcium and is also involved in many apoptotic pathways<sup>78</sup>.

Importantly, much research has shown that regulation of intracellular calcium by EPO plays a critical role in erythroid progenitor cell differentiation, proliferation and survival<sup>75,76</sup>. For example, treatment of ethylene glycol tetraacetic acid (EGTA), a nonspecific calcium chelator, can inhibit EPO-induced murine red cell colony growth, while treatment of an ionophore could enhance colony growth by increasing calcium

influx<sup>80</sup>. In a murine erythroleukemia cell line, it was demonstrated that an increase in calcium influx is required for the commitment of differentiation<sup>76,81</sup>. In CFU assays using human hematopoietic progenitors, increases of calcium concentration in response to EPO were observed during different stages of differentiation, suggesting that a calcium signal is required throughout erythropoiesis<sup>75</sup>. In our study, the *Dot1L* mutant yolk sac hematopoietic progenitors, which exhibit an erythropoietic defect, also show abnormal calcium influx when treated with EPO. Moreover, the ability of EPO to activate calcium influx and influence cell proliferation and survival has also been demonstrated in nonerythroid cells, such as myoblasts and brain tissue<sup>82</sup>. All of these studies suggest that EPO, as a growth factor, has a very broad role in maintaining proliferation and preventing apoptosis in different cell types, and highlight the importance of understanding the calcium influx mechanism by EPO.

It has been established that EPO causes a calcium influx through a transient receptor potential (TRP) protein superfamily, first identified in *Drosophila*<sup>83</sup>. Based on sequences from *Drosophila* TRP, a large number of mammalian isoforms were cloned and have been divided into six subfamilies. One of the subfamilies, TRPC, has been investigated in depth and was confirmed to be major players in EPO-mediated calcium influx<sup>83</sup>. In the mouse, it has been shown that among the six members of the family, TRPC1 to TRPC6, only TRPC2 and a certain amount of TRPC6 mRNA and protein are expressed in hematopoietic cell lines<sup>84</sup>. In this study, researchers used a CHO cell model system and showed that EPO is able modulate calcium influx through TRPC2. EPO, external calcium, TRPC2 and EPOR are all required for a calcium rise. The function of TRPC6 and its relationship to TRPC2 were investigated subsequently by the same group.

In this study, they first confirmed that EPO could regulate calcium influx in primary hematopoietic cells harvested from the spleens of phenylhydrazine-treated mice and showed that both TRPC2 and TRPC6 were expressed in these primary cells by RT-PCR, Western blotting, and confocal microscopy. They used transfected CHO cells as the model system, in which the TRPC composition can be controlled. They found that EPO stimulated a significant increase in calcium in cells transfected with TRPC2 isoforms, which was consistent with their previous study. But EPO did not induce a calcium influx in cells transfected with TRPC6. In cells transfected with both TRPC2 and TRPC6, these two protein factors were associated and calcium influx was inhibited. This indicates that the stoichiometry of expression of TRPC2 and TRPC6 is an important determinant by which hematopoietic cells regulate calcium influx in response to EPO stimulation<sup>85</sup>. A cell poised for response to EPO has a certain ratio of TRPC2 to TRPC6, and expression of these proteins is required for the normal response. Any change in the expression of either of these two factors will affect this ratio and result in altered calcium influx when the cells are exposed to EPO. The magnitude of the calcium response to EPO is thus regulated. This regulation has potential to affect the cellular response to EPO, with one ratio resulting in nominal responses, leading to normal erythropoietic development. Too little or too much of either of these channels can lead to too little or too great a calcium response, leading to overexpansion of an erythroid response or potential hematopoietic defects due to enhanced apoptosis of progenitor cells. In human cells, TRPC2 is a pseudogene, and EPO regulates calcium influx through TRPC3.

How does the EPO signaling pathway results in TRPC family activation and calcium influx? It turns out that the bridge between the two events is phospholipase C  $\gamma$

(PLC  $\gamma$ ). Several studies have shown that EPO stimulation of its receptor induces tyrosine phosphorylation and activation of PLC  $\gamma$ <sup>74,84</sup>. PLC  $\gamma$  activation produces IP<sub>3</sub>, which activates the IP<sub>3</sub> receptor (IP<sub>3</sub>R). IP<sub>3</sub>R then is able to interact with TRPC2. The association of IP<sub>3</sub>R and TRPC2 results in a conformational change of TRPC2 and opening of the calcium channel.

## 1.2 Histone H3 lysine 79 (H3K79) methylation by DOT1 methyltransferase

In eukaryotic cells, DNA is packaged within the nucleus along with histones and other nuclear proteins to form the nucleosome, the fundamental repeating unit of chromatin. The nucleosome is composed of a histone core octamer, made up of two each of H2A, H2B, H3, and H4 histones and a string of 146 basepairs of DNA wrapped around this histone core<sup>86</sup>. The histone proteins have a distinct globular domain with unstructured N- and C- terminal tails. The histone proteins (especially the tails) can be post-translationally modified in a variety of ways, including acetylation, phosphorylation, ubiquitination, and methylation. Among these modifications, acetylation and phosphorylation are reversible and seem to be associated with inducible regulation of genes, while histone methylation is more stable and seems to be transmitted in a heritable manner<sup>87</sup>. These modifications can influence chromatin structure, facilitate interactions between nucleosomes, and can potentially regulate transcription. Moreover, these modifications that occur at different sites of histones can create huge amount of diversity in terms of histone/nucleosome modifications, due to multiple combinations of different types of these enzymatic modifications on individual histone proteins<sup>87</sup>. Different proteins can bind specifically to the various forms of histone tails as a result of different

combinations of histone modifications. The binding of different protein factors results in distinct chromatin-dependent functions, such as gene activation or repression, or heterochromatin formation. This phenomenon was proposed as the “histone code hypothesis”. According to this hypothesis, different histone modifications can be translated to biological signals and act as signaling platforms to elicit downstream nuclear responses<sup>88</sup>.

Most of the histone modifications occur on histone tails because they are relatively unstructured and exposed to a variety of nuclear factors including enzymes that catalyze different states of modifications and other protein factors that interact specifically with different types of histone modification. Histone lysine methylation, which is one of the covalent histone modifications, exists in mono, di, and tri states<sup>87</sup>. One way that these methyl marks contribute to transcriptional regulation is to serve as a platform for the recruitment of effector proteins. The well-studied lysine methylation residues include K4, K9, K27, K36, and K79 of histone H3, and K20 of histone H4. In general, methylation at H3K9, H3K27, and H4K20 correlates with transcriptional repression, while methylation at H3K4, H3K36, and H3K79 correlates with transcriptional activation<sup>88</sup>. In addition to its role in transcriptional regulation, methylation has also been linked to X inactivation, cell fate determination, terminal differentiation, and the spatiotemporal patterning of Hox genes. Moreover, aberrant histone methylation has also been linked to various human cancers<sup>87</sup>. Histone lysine methylation is catalyzed by one family of histone lysine methyltransferases (HLMT).

#### 1.2.1 The characteristics and protein structure of DOT1.

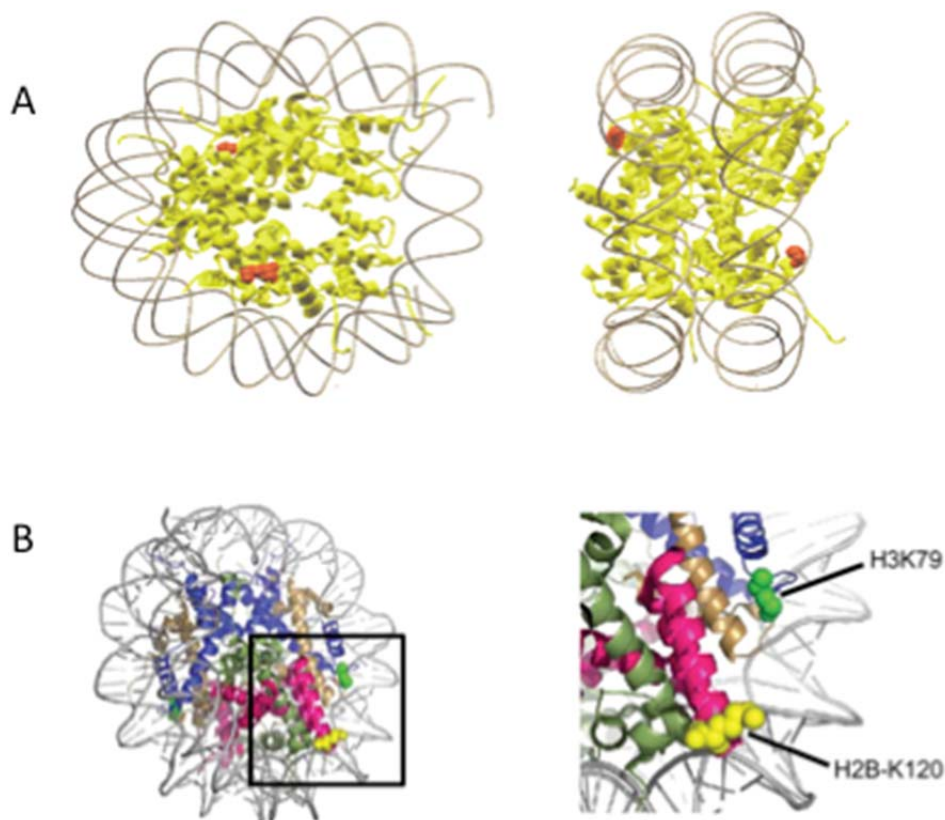
Most histone lysine methyltransferases contain a conserved SET (suppressor of

variegation, enhancer of zeste, and trithorax) domain, which is required for enzymatic activity and is believed to be a signature motif for this class of enzymes. In contrast, DOT1, the H3K79 methyltransferase, is quite different from most of HLMT family members. In the first place, unlike other histone lysine methyltransferases, DOT1 family members do not have a SET domain. Instead, their catalytic domain contains conserved sequence motifs characteristic of class I methyltransferases such as DNA methyltransferases (DNMTs) and the protein arginine methyltransferase PRMT1<sup>89,90</sup>. In a previous study, the crystal structure of human homolog of DOT1, hDOT1L, was determined at 2.5Å resolution. First, the researchers found that the N-terminal  $\alpha$ -helical domain of hDOT1L contains the HLMTase catalytic activity. Although the full-length hDOT1L contains 1537 amino acids, only the N-terminal ~360 amino acid share significant sequence homology with other species, such as yeast DOT1 and Drosophila homolog. Within this region, they found that amino acids 1-416 contain methyltransferase activity in an in vitro assay. Second, they found hDOT1L contains a S-adenosyl-L-Methionine or SAM binding motif, instead of a SET signature domain of other HLMTases. SAM binding motifs are characteristic of protein lysine and arginine methyltransferases. During the methyltransferase reaction, SAM donates a methyl group to a lysine or arginine amino acid. In proximity of SAM binding motif, they also found a potential lysine binding channel, through which lysine 79 of H3 could access the active site and is brought close SAM group. Finally, a disordered C-terminal region was shown to be important. Within this region, amino acids 391-416, which are enriched with positively charged residues, are crucial for HLMT activity. In the following gel mobility shift assays, this region showed important nucleosome binding function and it was

required for normal enzymatic activity<sup>90</sup>. Due to the structural similarity between DOT1 and arginine methyltransferases, it has been speculated that whether DOT1 possesses the ability of arginine methylation. However, this function has failed to be proven even though people put in extensive efforts using the most advanced technologies.

Secondly, the substrate, H3K79 is located at the loop 1 region between  $\alpha$  1 and  $\alpha$  2 helices within the globular domain of histone H3 and exposed on the nucleosome surface and do not interact with DNA or other histones, as revealed by an X-ray crystal structure analysis<sup>8,90,91</sup> (Figure 1.4 A). This region is also close to the interface between H3/H4 tetramer and H2A/H2B dimer<sup>86</sup>. Therefore H3K79 is positioned in a place where it can influence the access to the interface, suggesting the possibility that H3K79 methylation may regulate the interaction of protein factors to chromatin, such as silencing proteins. For example, Sir proteins (silent information regulator) as well as other silencing proteins and chromatin remodeling complexes, which are important for folding of nucleosomes into silent chromatin, may be regulated by H3K79 methylation.

When the enzymatic activity of DOT1 was studied, it showed that DOT1 preferentially methylated H3K79 in the context of nucleosomes rather than core histones or recombinant H3<sup>10</sup>. This observation suggested that DOT1 not only recognizes H3K79, but may also recognize other conformational features of the nucleosomes. As previously discussed, H3K79 is located at the loop 1 region of histone H3 within the globular domain and exposed on the nucleosome surface. It also lies in close proximity to the interface between the H3/H4 tetramer and H2A and H2B dimer. Based on the observation that DOT1 preferentially works on nucleosomes and specific position of H3K79, it has



**Figure 1.4 The location of H3K79 on the nucleosome.** (A) The structure of the yeast nucleosome core particle, viewed down the superhelical axis (left) and side view (right). H3K79 is shown in red space filling, while the histone protein main chains are represented as yellow ribbons. Gray lines indicate DNA. (B) The crystal structure of the human nucleosome (PDB code 3AFA) is shown on the left. Histone H2A is shown in light green, H4 is shown in sand, H2B is shown in hot pink, and H3 is shown in blue. A close-up view of the nucleosome surface is on the right, with the location of H2B-K120 shown as yellow spheres and H3K79 shown as green spheres. Note that H2B-K120 and H3K79 are located in proximity on the same exposed surface of the nucleosome. Panel A is from van Leeuwen et al, 2002<sup>10</sup>. Panel B is from Nguyen et al, 2011<sup>92</sup>.



been suggested that DOT1 function is regulated by trans-histone cross-talk. X-ray crystal structure study showed that H3K79 and H2B-K123 are adjacent on the same exposed nucleosome surface (Figure 1.4 B). H2B-K123 can be mono-ubiquitinated by the ubiquitin-conjugating E2 enzyme Rad6 and ubiquitin E3 ligase Bre1. It would be interesting to determine whether there is a cross-talk between the modifications on H3K79 and H2B-K123. Importantly, the deletion of Rad6 not only abolished H2B ubiquitination, but also destroyed H3K4 and H3K79 methylation. Mutation of H2B-K123 also resulted in lack of H3K4 and H3K79 methylation, indicating that H2B ubiquitination is required for H3K4 or H3K79 methylation. In contrast, the deletion of DOT1 does not influence H2B-K123 ubiquitination, suggesting that H2B ubiquitination is the prerequisite for DOT1 function, and works upstream of the trans-histone pathway<sup>93</sup>. Then how does H2B-K123 mediate H3K79 methylation? So far, there are three possible mechanisms and they are not mutually exclusive. 1). H2B-K123ub can interact with DOT1 indirectly through some other protein(s). In support of this, studies showed that H2Bub can recruit protein factors Rpt4 and Rpt6 to chromatin to facilitate H3K79 methylation. 2). H2B-K123ub can interact with DOT1 directly. It has been shown that N-terminal of DOT1 contains a lysine-rich region, through which DOT1 can interact directly with ubiquitin. Deletion of this region resulted in failure of nucleosome binding and diminished di- and trimethylation of H3K79. 3). Ubiquitination of H2B results in chromatin structure changes to facilitate DOT1 function. Evidence is that ubiquitination of H2B can disrupt chromatin compaction and interchromatin fiber interactions to increase substrate accessibility<sup>92</sup>. Current literature supports the notion that all of these

nonexclusive mechanisms work together to facilitate H3K79 methylation mediated by H2B ubiquitination.

Although H3K79 methylation process has been extensively studied, the mechanism of demethylation of this mark is still largely unknown. So far, at least one histone demethylase exists for each known histone lysine residues subject to methylation, except for H3K79. But there is already some evidence suggesting that this elusive H3K79-specific demethylase does exist. For example, in both yeast and human cells, the H3K79 di-methylation level fluctuates with cell cycle<sup>89,94</sup>. Also, H3K79 di-methylation level reduced during early embryonic development in mice, suggesting the existence of a demethylase in mature oocytes<sup>95,96</sup>. It should not take very long before the identity of the enzymes(s) is unveiled.

#### 1.2.2 Important functions of DOT1

As we discussed before, DOT1 is a histone lysine methyltransferase that is responsible for H3K79 methylation. H3K79 methylation is conserved between different species, from yeast to human. There are three types of methylation on this site: mono-, di-, and tri- methylation. So far, the DOT1 is thought to be the sole enzyme responsible for these modifications in all species. Mutation of *Dot1* in different model system results in specific ablation of H3K79 methylation, while modifications on other sites of histone are not affected in general<sup>7,89,92</sup>. Therefore DOT1's function in cells is thought to be non-redundant. Through modifications on H3K79, tremendous cellular and physiological function can be regulated by DOT1.

DOT1 was initially identified in *Saccharomyces cerevisiae* as a disruptor of telomeric silencing<sup>97</sup>. In subsequent studies, both over expression and inactivation of

DOT1 or mutations at H3K79 lead to loss of telomeric silencing<sup>10</sup>. A possible explanation came from the nuclear redistribution of Sir2 and Sir3 proteins in *Dot1* mutant yeast. Telomeric silencing is critically dependent on the Sir proteins, which are recruited to telomeres by DNA binding protein Rap1 and other associated protein factors<sup>10</sup>. Chromatin immunoprecipitation analyses provided evidence that Sir2 and Sir3 association with telomeric DNA is diminished in the *Dot1* mutant, and this effect is even more significant when H3K79 is mutated. However at the same time, in the subtelomeric region, Sir2 and Sir3 binding was increased in *Dot1* mutant<sup>10</sup>. It seems that Dot1 is not required for Sir proteins binding, but precludes their binding. Based on these observations, a model was generated in which H3K79 methylation limits Sir protein binding and silencing to discrete loci, while it precludes their binding elsewhere in the genome. According to this model, *Dot1* mutation results in elimination of H3K79 methylation, leading to promiscuous binding of Sir proteins in genome and titrating the limited pool of Sir proteins away from telomeres. Over expression of DOT1 results in hypermethylation of H3K79 and leaves little unmodified histone H3 in the nucleus, which also impairs telomere-Sir protein association and a general loss of silencing throughout the genome. Under both conditions, telomeric silencing is disrupted<sup>98-100</sup>. On the other hand, another member of Sir protein family, Sir3 has been shown to inhibit H3K79 methylation by competing with DOT1 for binding to the same basic patch (R<sub>17</sub>H<sub>18</sub>R<sub>19</sub>) of the histone H4 tail, which is required for DOT1 function<sup>101,102</sup>. Thus, the balance of Sir protein amount and level of H3K79 methylation is very important for heterochromatin formation and telomeric silencing maintenance.

Besides its important function in telomere silencing, genome-wide analysis also connects H3K79 methylation with active transcription. Some early studies in yeast showed that about 10% of the genome is enriched with Sir proteins and hypomethylated at H3K79. These regions correspond to low levels of transcription. In contrast, 90% of the yeast genome is highly methylated at H3K79 and these regions are actively transcribed<sup>10,103</sup>. This correlation has also been observed by other groups, showing that H3K79 methylation can be found at active V(D)J recombination sites using mammalian cell lines<sup>104</sup>. Despite these initial works, the following studies with the help of high-throughput technology to map specific chromatin modifications on a genome-wide scale have generated inconsistent results. Some research supported this positive correlation between H3K79 methylation and transcription. For instance, in *Drosophila*, chromatin immunoprecipitation coupled with gene expression microarray (ChIP-chip) confirmed the correlation by showing that H3K4 and H3K79 methylation are associated with active gene transcription<sup>105</sup>. In contrast, some other research showed different results. For example, in two studies, ChIP-chip assays were also carried out in yeast and human T cells. But they did not find a significant correlation between H3K79 methylation and transcriptional activity<sup>106,107</sup>. Several important caveats to these data have been described, and these may alter the interpretation of the results of these studies. First, the authors relied exclusively on micrococcal nuclease digestion of chromatin and it was shown that sometimes this method does not work as efficiently as sonication. Second, they performed ChIP in the absence of sodium dodecyl sulfate (SDS). SDS results in partial denaturation and exposure of H3K79 methylation, which is required for detection within formaldehyde-cross-linked chromatin<sup>92</sup>. After that, several studies came out in support of

this positive correlation. In 2008, Steger et al. observed the ubiquitous nature of DOT1L recruitment and H3K79 methylation at actively transcribed sites. A strong similarity between the patterning of H3K79 methylation and that of H3K4 methylation in mammalian chromatin was identified, which suggests that parallel pathways may exist to specify gene activity or antagonizing gene silencing. Also they found that H3K79 mono-methylation is enriched in intergenic regions and is usually associated with regulatory proteins, suggesting H3K79 mono-methylation may demarcate gene-regulatory elements in the genome<sup>13</sup>. Findings reported in this report from our laboratory are consistent with this positive correlation. In the G1E mouse cell line, we demonstrated H3K79 mono-, di- and tri-methylation are all enriched in the important regulatory regions, such as the promoter and enhancers in the *Gata-2* locus, which is actively transcribed. In contrast, on the silenced *pu.1* locus, there is only H3K79 mono-methylation observed at the promoter and upstream regulatory elements. No H3K79 di and tri-methylation enrichment are observed in these regions<sup>7</sup> (see Figure 4.3). Collectively, analyses from yeast, mouse, *Drosophila*, and human genomes indicate that H3K79 methylation is indeed associated with active transcription.

An interesting phenomenon is that although H3K79 methylation is a universal histone modification that can be found throughout the genome, the knockout of *Dot1* only results in transcriptional changes in a subset of genes, but not all of them. For instance, in Steger's study in 2008, they found that although there is strong correlation between H3K79 di- and tri-methylation and transcriptional activity, many active genes can still be transcribed at normal levels in the absence of H3K79 methylation in their transcribed region. Another study using embryonic stem (ES) cells investigated the consequence of

*Dot1L* deficiency in the transcriptome. They found that under both standard ES cell culture condition and after 3-days of retinoic acid (RA)-induced differentiation, *Dot1L* deficiency resulted in expression changes in a group of genes in the whole genome, most of which have known functions in the cell cycle, cellular proliferation and differentiation<sup>108</sup>. In our research, which is focused on *Dot1L* function in embryonic hematopoiesis, we analyzed expression change of genes important in hematopoiesis in *Dot1L* KO progenitor cells using RT-PCR. We found that only some critical transcription factors and a voltage-independent calcium channel change their transcription level upon *Dot1L* KO, while most other genes, including hemoglobin genes and cytokine receptors, are not affected by *Dot1L* loss<sup>7</sup>.

DOT1 plays important role in the DNA damage response. The first evidence for this came from a study showing that H3K79 methylation can interact with the tudor domain of human 53BP1 protein and recruit it to DNA double strand breaks (DSBs). *Dot1L* knock down or mutation of tudor domain of 53BP1 both inhibited 53BP1 recruitment to DSBs. This is a conserved mechanism since this phenomenon can be observed from yeast to mammalian cells<sup>109</sup>. Later on, studies showed that DOT1 is also required for the DNA damage checkpoint response. When yeast cells are treated with ionizing radiation (IR), they usually undergo a G1 checkpoint delay, a surveillance mechanism allowing time for DNA repair. *Dot1* mutant yeast cells are defective in G1 and intra-S phase checkpoint<sup>11</sup>. Importantly, mutations that may affect DOT1 function also results in the same defect. This includes Rad-Bre1 pathway required for H2B ubiquitination, mutation of H2B lysine 123, or mutation of H3K79. The molecular mechanism is that loss of DOT1 prevents the binding of the yeast 53BP1 ortholog Rad9

to DSBs, which in turn prevents the recruitment and activation of Chk2 homolog Rad53 kinase, a key step in DSB repair pathway<sup>110-112</sup>. DOT1 was also found to be required for other types of UV-induced DNA damage besides DSB and meiotic checkpoint control in yeast<sup>110,113</sup>. However, the knowledge about DOT1L's function in DNA damage checkpoint control in mammalian is quite limited.

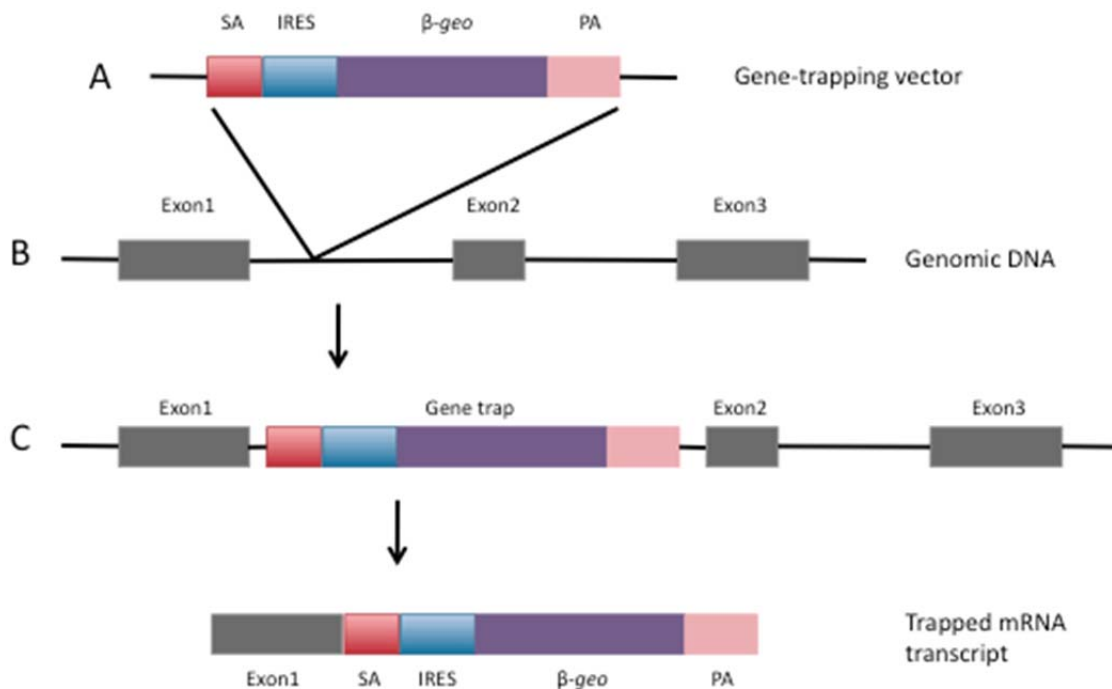
DOT1's function is not only investigated in cell lines and lower eukaryotes, *Dot1L* deficient mouse lines have been established by several groups to study its biological function in the whole organism. In 2008, Jones, et al. generated a mouse line in which exon 5 and 6 of *Dot1L* gene encoding 108 amino acids that form several conserved motifs in the catalytic domain were deleted. They showed for the first time that *Dot1L* is essential for mouse embryonic development and *Dot1L* deficiency results in embryonic lethality. At E8.5, no distinguishable defect was observed between *Dot1L* KO and WT control. By E9.5 *Dot1L* KO embryos are smaller than littermates, have severe cardiac dilation and stunted tails upon gross observation under a dissecting microscope. By E10.5, the percentage of viable *Dot1L* KO is much lower than the expected Mendelian ratio, and no *Dot1L* KO embryos survive beyond E10.5. They also derived ES cells from this *Dot1L* KO mouse line and showed that *Dot1L* deficiency results in ES cell growth defects, telomere elongation, aneuploidy, and loss of heterochromatin marks at telomeres and centromeres<sup>12</sup>. In order to further study *Dot1L*'s function in fetal heart development, a cardiac-specific *Dot1L* KO mouse model was generated. When *Dot1L* was only deficient in cardiac system, increased mortality rate with chamber dilation, increased cardiomyocyte cell death, systolic dysfunction, and conduction abnormalities were observed. Importantly, similar defects are also obvious in patients with dilated

cardiomyopathy. Mechanistic studies showed that the transcription of dystrophin is regulated by DOT1L-mediated H3K79 methylation. The stability of dystrophin-glycoprotein complex is important for cardiomyocyte viability and dystrophin may be a major target mediating DOT1L function in cardiomyocytes<sup>114</sup>. This study not only revealed the mechanism by which *Dot1L* performs its function in cardiac system, but also raised the possibility of diagnosis and treatment of certain human heart diseases.

The studies in our laboratory were based on a mouse line in which a gene trap was used to abrogate *Dot1L* activity (Figure 1.5). Our results were consistent with the previously described study that DOT1L function is essential for embryogenesis, with embryonic lethality occurring at mid-gestation in *Dot1L* deficient mice. Importantly, we found that a defect in early erythropoiesis is attributed to embryonic lethality. We also reported that in hematopoietic progenitor cells of these embryos, two genes critical for early hematopoietic fate decision, *Gata-2* and *pu.1*, were differentially affected by the loss of *Dot1L* function<sup>7</sup>. Consistent with our results about *Dot1L* function in hematopoiesis, recently another group using a conditional knockout model showed that *Dot1L* is also required for murine postnatal hematopoiesis. By doing flowcytometry and transplantation experiments, they showed that deletion of *Dot1L* leads to pancytopenia and failure of hematopoietic homeostasis, and *Dot1L* deficient cells are incapable of reconstituting recipient bone marrow<sup>115</sup>. This research is a good extension of our *Dot1L* study and their results are meaningful. But the researchers did not provide any mechanistic explanation about *Dot1L*'s function in postnatal hematopoiesis.

In addition to its role in normal hematopoiesis, DOT1L is also strongly associated with leukemias resulting from translocation of MLL gene, in which MLL is





**Figure 1.5 Illustration of gene trap.** (A) Typical gene trapping vector, showing the splice acceptor (SA), the internal ribosomal entry site (IRES), the  $\beta$ -geo gene (fusion of the  $\beta$ -galactosidase gene and the neomycin-resistance gene), and the polyadenylation site (PA). (B) Conventional genomic DNA, showing exons and introns. (C) Resultant mRNA after gene trapping insertion between Exon1 and Exon2. On transcription to form mRNA, the SA allows the vector to be spliced to exon1. Transcription is terminated before exon2 because of the existence of a poly-A site on the vector. The presence of the IRES ensures the translation of the  $\beta$ -geo marker.

fused in frame with dozens of translocation partners. The misregulation of DOT1L is achieved because many MLL translocation partners, including AF9, ENL, AF4, AF10, EAP, SEC, etc., can interact with DOT1L, therefore DOT1L is recruited to MLL fusion protein target genes<sup>116</sup>. For example, in MLL-AF10 transformed cells, both MLL-AF10 fusion protein and H3K79 di-methylation were enriched at Hoxa9, which is important in leukemogenesis and is a target of MLL-AF10. DOT1L enzymatic activity is required for Hoxa9 gene overexpression<sup>116</sup>. DOT1L has also been shown to be required for both initiation and maintenance of MLL-AF9 mediated leukemogenesis in vivo and in vitro. In this study, the researchers indicated that the recruitment of DOT1L to MLL-AF9 target genes, enrichment of H3K79 methylation on these locus, and up-regulation of important oncogenes, such as Hoxa and Meis1 constitute the molecular basis of MLL-AF9 induced leukemogenesis<sup>117,118</sup>. Collectively, current data support a general mechanism by which DOT1L contribute to leukemogenesis process: interaction between DOT1L and MLL fusion partners results in abnormal recruitment of DOT1L to the gene targets of MLL fusion proteins, some of which are critical oncogenes. The subsequent aberrant H3K79 methylation enrichment leads to constitutive over expression of these genes, which in turn facilitates leukemic transformation. Based on this assumption, it may be possible to treat certain types of leukemia if DOT1L enzymatic activity or DOT1L interaction with MLL fusion proteins is specifically inhibited<sup>119</sup>. But due to DOT1L's role in normal hematopoiesis, the possible side effect should be taken into account before this method is applied to leukemia patients.

In summary, DOT1 is a histone lysine methyltransferase with both unique structure and substrate specificity. It plays critical role in telomere silencing maintenance

and is capable of regulating transcription at both heterochromatin and euchromatin. Generally, H3K79 is enriched at actively transcribed gene loci. In addition to transcription, DOT1 plays an important role in DNA damage checkpoint response. Moreover, DOT1L in mammals is required for embryogenesis, cardiac development and hematopoiesis. It also plays a pivotal role in MLL-induced leukemogenesis. However, it turns out that activity of DOT1 and its associated H3K79 methylation is extremely complicated. Besides its function in cardiovascular and hematopoietic systems, DOT1's function in other aspects needs to be elucidated. A thorough characterization of DOT1 function will not only help us understand its essential role in normal biological process, but will also provide us a potential avenue to the treatment of certain human diseases. In chapter 2, materials and experimental methods used in my study are described. In chapter 3, the erythropoietic defects in *Dot1L* mutant yolk sac cells are discussed. In chapter 4, two hypotheses are discussed. My first hypothesis is that DOT1L is required for maintaining the normal level of *Gata-2* and *pu.1* in the hematopoietic progenitor cells. When *Dot1L* is mutated, it will directly result in transcription pattern change of *Gata-2* and *pu.1*, which in turn causes specific erythroid development defect. My second hypothesis is that *Dot1L* deficiency results in down regulation of *Trpc6*. The change of stoichiometry of TRPC6 and TRPC2 in turn results in abnormal calcium influx in response to EPO during erythropoiesis, which leads to specific erythropoietic defect. Experimental results are described and discussed. Chapter 5 is used for summarization of previous results and discussion for future directions.

## Chapter 2

### Materials and Methods

#### 2.1 Standard solutions and reagents:

##### **mEPO**

1X10<sup>5</sup> units/ml mEPO (PeproTech) in water with 0.1%BSA, stored at -20°C.

##### **SCF**

10 ug/ml SCF (R&D) in sterile PBS containing 0.1%BSA, stored at -20°C.

##### **Puromycin:**

1mg/ml in water, stored at -20°C.

##### **Amphicilin (stock solution):**

100mg/ml in 50% water and 50% ethanol.

##### **Roswell Park Memorial Institute medium (RPMI) complete medium (for K562 cells growth):**

RPMI 1640 with 1% sodium pyruvate, 1% non-essential amino acid, 1% L-glutamine, 10% FBS, and 1% penicillin/streptomycin, filter sterilized, stored at 4°C.

##### **Isocove's Modified Dulbecco's Medium (IMDM) complete medium (for G1E cells growth):**

IMDM with 0.01% monothioglycerol, 1% penicillin/streptomycin, 15% FBS, mEPO 2U/ml, SCF 100ng/ml, filter sterilized, stored at 4°C.

##### **Methylcellulose medium for primitive erythropoiesis:**

Methylcellulose (R&D Systems) with 10% plasma-derived serum (Antech Inc), 5% protein free hybridoma medium (Gibco-BRL), and cytokines SCF (100ng/ml) and mEPO (2U/ml), stored at -20°C.

##### **Methylcellulose medium for definitive hematopoiesis:**

MethCult<sup>®</sup> GF3434 methylcellulose medium with recombinant cytokines for mouse cells (StemCell Technologies), stored at -20°C.

**Methylcellulose medium for definitive erythropoiesis:**

MethCult<sup>®</sup> SF3334 methylcellulose medium with recombinant cytokines for mouse erythroid progenitor cells (StemCell Technologies), stored at -20°C.

**Yolk sac digestion solution:**

PBS containing 20% FBS and 0.1% collagenase (StemCell Technologies), stored at 4°C.

**Benzidine staining stock solution:**

100 mg Benzidine (Sigma-Aldrich) suspended in 50ml 0.5M acetic acid, stored at 4°C.

To make working solution: add 20 ul 30% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into 1ml of the Benzidine stock solution, then mix with 4 ml Dulbecco's Modified Eagle Medium (DMEM) medium. Freshly made before experiment.

**Hemin stock solution (for K562 cell differentiation):**

4mM hemin, 0.02mM NaOH, 0.05mM Tris·HCl pH7.8, filter sterilized and stored at -20°C. Working concentration is 50uM.

**TBS buffer 10X:**

24.23 g Trizma HCl, 80.06 g NaCl, suspended in 800 ml ultra pure water. Adjust pH to 7.6 with HCl and top up to 1 liter.

**TBST:**

100 ml TBS 10X+900 ml ultra pure water+1ml Tween20

**Lysis buffer for Western Blot:**

150 mM sodium chloride

1.0% NP-40 (Triton X-100 can be substituted for NP-40)

50 mM Tris, pH 8.0

**Loading buffer for Western Blot:**

NuPAGE® LDS sample buffer 4X (Life Technologies)

**Running buffer for Western Blot:**

1X Tris-glycine buffer containing 25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3.

**Transfer buffer for Western Blot:**

The same as running buffer with the addition of methanol to a final concentration of 20%.

**SDS lysis buffer:**

1% SDS, 10mM EDTA, 50mM Tris·HCl pH8.0, stored at room temperature.

**ChIP dilution buffer:**

0.01%SDS, 1.1%Triton X-100, 1.2mM EDTA, 167mM NaCl, 16.7mM Tris·HCl pH8.0, stored at room temperature.

**Low salt immune complex wash buffer:**

0.1%SDS, 1%Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris·HCl pH8.0, stored at room temperature.

**High salt immune complex wash buffer:**

0.1%SDS, 1%Triton X-100, 2mM EDTA, 500mM NaCl, 20mM Tris·HCl pH8.0, stored at room temperature.

**1X TE buffer:**

10mM Tris·HCl pH8.0, 1mM EDTA, stored at room temperature.

**LiCl immune complex wash buffer:**

0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris·HCl pH8.0, stored at room temperature.

## 2.2 Animal Husbandry

### 2.2.1 Generation of *Dot1L* mutant mice and breeding scheme

A mouse ES cell line (Sequence Tag, RRR032) that contains a gene-trap integration within exon 13 of the *Dot1L* gene and a  $\beta$ -geo selection cassette was obtained from Bay Genomics. *Dot1L*-mutant mice were generated by using the recommended protocols of Bay Genomics for blastocyst injection and mouse breeding (<http://baygenomics.ucsf.edu/protocols/comp1/blastocyst.html>). *Dot1L* heterozygous (*Dot1L*<sup>+/-</sup>) F1 mice were generated by breeding chimeras with C57B1/6 females (The Jackson Laboratory). Stocks of heterozygous mice were maintained by continuous backcrossing to C57B1/6 stocks. Generation of *Dot1L*<sup>-/-</sup> embryos was achieved by crossing male *Dot1L*<sup>+/-</sup> and female *Dot1L*<sup>+/-</sup>.

### 2.2.2 Genotyping of progeny

The genotypes of all offspring were analyzed by PCR on genomic DNA isolated from ear punches, tail tips, or embryonic tissue depending on the intended protocols. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen). The presence of the gene trap was tested with the primers (*Dot1L* Forward 5'-TGGACACTTACCCAGCACTTCC-3', Wild Type reverse 5'-GAGGGAGATGGCTTTTGTACAGTAG-3', *Dot1L* KO reverse 5'-TTTGAGCACCAGAGGACATCCG-3'). The wild type allele gives rise to a band of 326-bp band. The *Dot1L* KO allele containing the gene trap gives rise to a band of 271-bp band. The heterozygous progenies generate both bands.

### 2.2.3 Animal euthanasia

All mice used in this study were sacrificed by asphyxiation with carbon dioxide followed by cervical dislocation to confirm mortality or by direct cervical dislocation according to IACUC standard operating procedure (SOP).

## 2.3 DNA manipulations

### 2.3.1 Genomic DNA isolation

Genomic DNA was isolated from mouse and embryonic tissues using the DNeasy Blood and Tissue Kit (Qiagen). Briefly, tissues from mouse ear or tail tips and embryonic tissue were incubated overnight in 180  $\mu$ l ATL buffer (tissue lysis buffer) and 20  $\mu$ l proteinase K. The following morning the samples were vortexed and 200  $\mu$ l buffer AL and 200  $\mu$ l absolute ethanol were added to the samples and mixed thoroughly. The entire sample was pipetted into DNeasy Mini spin column and centrifuged at 6000 x g for 1 minute. Next, 200  $\mu$ l buffer AW1 (wash buffer 1) was added to the column and again centrifuged for 1 minute at 6000 x g. Then 500  $\mu$ l buffer AW2 (wash buffer 2) was added and centrifuged at 20,000 x g for 3-5 minutes. The column was placed in a clean 1.5 ml Eppendorf tube and appropriate amount of buffer AE (elution buffer) was added directly onto the column membrane. The sample was incubated at room temperature for 1 minute and then centrifuged for 1 minute at 6000 x g to elute. DNA samples were frozen at -20°C until use.

### 2.3.2 Polymerase Chain Reaction (PCR)

4X primer stock solution was obtained by dissolving PCR primers (Integrated DNA Technologies, Inc) in pure water to a concentration of 100  $\mu$ M (100 p Mole/ $\mu$ l). Primers were stored at -20°C. PCR reactions were carried out on 2720 Thermal Cycler (Applied Biosystems). PCR products were analyzed in 2% agarose gels.



A typical PCR cocktail:

10X Tsg reaction buffer 2.5 ul

25 mM magnesium chloride 3 ul

dNTPs 1 ul

Tsg DNA polymerase 0.2 ul

Primer (forward) 0.5 ul

Primer (reverse) 0.5 ul

Water 15.3 ul

Template 2 ul

Total 25 ul

Typical conditions for PCR (annealing temperature and cycle number varied):

95°C 5 minutes

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94 °C 45 seconds

61°C 45 seconds            30 cycles

72 °C 100 seconds

-----

72 °C 10 minutes

4°C hold

## 2.4 RNA manipulations

### 2.4.1 RNA isolation

RNA extraction using TRIzol® (Invitrogen) was performed following standard protocol. Cells to be analyzed were washed with PBS. Then the cells were centrifuged at

2300 rpm for 3 minutes at 4°C and discarded the supernatant. 1ml TRIzol per  $1 \times 10^6$  was added to cell pellet and an 18 gauge syringe was used to disrupt the pellet. The samples were incubated at room temperature for 3 minutes. The cellular debris was removed by centrifugation at 12,000 g for 15 minutes at 4°C. The supernatant was transferred to 1.5 ml Eppendorf tubes and equal volume of isopropanol was added to precipitate the RNA. The samples were incubated at room temperature for 15 minutes and then centrifuged at 15,000 g for 15 minutes to pellet the RNA. Supernatant was discarded and the RNA was washed in 70% ethanol. Samples were centrifuged at 15,000 g for 15 minutes and supernatant was discarded. Finally the RNA pellet was resuspended in appropriate amount of water.

#### 2.4.2 Reverse Transcriptase (RT)-PCR

cDNA was generated using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). For a single reaction, 5X VILO™ Reaction mix 4ul, 10X SuperScript® Enzyme mix 2ul, RNA (up to 2.5 ug) and water were mixed to total volume of 20ul. The sample was incubated at 25°C for 10 minutes and then incubated at 42°C for 60 minutes. The reaction was terminated at 85°C for 5 minutes. cDNA samples were stored at -20°C.

#### 2.4.3 Real-time PCR for gene expression analysis

Sequences of real-time PCR primers were obtained from either Primer Bank (<http://pga.mgh.harvard.edu/primerbank/>) or designed with MacVector software (MacVector Inc.). Quantitative real-time PCR was performed and analyzed with the 7500 real-time PCR system (Applied Biosystems). Fold change was calculated by the  $\Delta \Delta C_t$  method. Gene expression was normalized to GAPDH.

### 2.5 Preparation of single yolk sac cell suspension

Yolk sacs from embryos at ages E8.5 or E10.5 were used in my research. Embryos were obtained from timed mating between heterozygous Dot1L-mutant mice. Yolk sacs were incubated in 0.1% collagenase (StemCell Technologies Inc) in PBS with 20% FBS at 37°C for 30 minutes. The digested yolk sacs were aspirated through 25- and 27-gauge needles sequentially and then filtered through a 70- $\mu$ M nylon strainer (BD Biosciences). Genotyping was performed on DNA isolated from corresponding embryo tissues.

## 2.6 Culture of mouse embryonic fibroblasts

E10.5 embryos were dissociated by aspiration through a 25-gauge needle. Cells were incubated in 12-well plates at 37°C in complete Dulbecco modified Eagle medium (DMEM)/10% FBS until confluent. Mouse embryonic fibroblasts (MEF) were then incubated in DMEM with 0.1% FBS for 72 hours, restimulated with DMEM with 20% FBS for 24 hours, and harvested for analysis.

## 2.7 Extraction of histone from both starved and restimulated MEF

MEF were washed twice with ice-cold PBS with 5mM sodium butyrate then trypsinized and resuspended ( $10^7$  cells/ml) in Extraction Buffer (TEB: 0.5% Triton X-100, 2mM phenylmethylsulfonyl fluoride, 0.02%  $\text{NaN}_3$  in PBS). The MEF/TEB suspensions were incubated on ice for 10 minutes and centrifuged at 800g for 10 minutes at 4°C. The supernatant was discarded, and the pelleted nuclei were washed in one-half the volume of TEB and centrifuged as before. The nuclear pellets were resuspended in 0.2N HCL at a density of  $4 \cdot 10^7$ /mL at 4°C overnight. The samples were centrifuged (800g), and supernatant was analyzed. Protein content was determined by the Bradford assay (Bio-Rad), and aliquots were stored at -80°C.

## 2.8 Whole-mount immunolabeling for fluorescence

Embryos were dissected at E10.5 in PBS. The uterus and decidua were carefully removed and discarded. The yolk sac was separated from the embryo by severing the vitelline arteries and the embryo was used for genotyping. The yolk sacs were collected in separate wells in 24-well plate and then fixed in 1mL of 3% paraformaldehyde/PBS for 15 minutes on ice. Yolk sacs were permeabilized in PBS containing 0.2% Triton X-100 for 30 minutes. Nonspecific binding was blocked by incubation with 3% BSA plus 5% donkey serum in PBS for one hour at 20°C. Tissues were then incubated with anti-platelet endothelial cell adhesion molecule 1 (CD31, BD PharMingen) at 4°C overnight, then washed 5 times in 0.5 mL blocking solution for one hour each. A fluorescein isothiocyanate-coupled donkey anti rat secondary antibody (1:75 dilution in blocking solution, 4°C overnight) was used for detection (Jackson ImmunoResearch Laboratories Inc). The yolk sacs were then mounted onto glass slides with the use of an anti-photobleaching medium for immunofluorescence detection.

## 2.9 Analysis of primitive and definitive hematopoiesis by colony forming unit assay

Primitive erythropoiesis was measured by performing colony-forming unit assay. Briefly, E8.5 yolk sacs were dissected and dissociated into single cell suspension. Equal number of cells from each yolk sac ( $3 \times 10^4$ ) were plated in methylcellulose containing 10% plasma-derived serum (Antech Inc), 5% protein free hybridoma medium (Gibco-BRL), and cytokines KL (100 ng/ml) and EPO (2 U/ml). Colonies were identified, counted and measured after 5-7 day culture in incubator with  $\geq 95\%$  humidity, 5% CO<sub>2</sub> and 37°C. In order to analyze definitive hematopoiesis, erythroid burst-forming unit (BFU-E) and granulocyte, macrophage colony-forming unit (CFU-GM) assays were

performed. E10.5 yolk sacs were dissociated into single cells and  $5 \times 10^4$  cells from each yolk sac were plated in M3434 methylcellulose medium (StemCell Technologies) in 35-mm culture dishes. Cell culture was incubated at 37°C, 5% CO<sub>2</sub>, with  $\geq 95\%$  humidity for 10-12 days. In some experiments, cells were plated on medium (M3334) that only supports the growth of erythroid lineage (CFU-E and mature BFU-E colonies) and cultured for 2-3 days for colony identification and counting.

Cultures were viewed with a NIKON ECLIPSE TS100 microscope equipped with 10X to 20X phase contrast, numerical aperture 1.2 objective lens. Images were taken with a LEICA DFC480 camera with the use of Leica Firecam software at room temperature. Single-colony area was measured with ImageJ software (National Institutes of Health).

## 2.10 Benzidine staining of erythroid colonies or hemoglobin-containing K562 cells

A benzidine solution containing 0.6% (wt/vol) benzidine base, 2% (vol/vol) hydrogen peroxide, and 12% (vol/vol) acetic acid was used. For observation of primitive erythroid colonies derived from E8.5 yolk sacs and BFU-E colonies derived from E10.5 yolk sacs, benzidine solution was directly applied on the surface of methylcellulose medium for 5 minutes. Then the stained colonies were viewed with a NIKON ECLIPSE TS100 microscope equipped with 10X to 20X phase contrast objective lens. For K562 cell observation, cells were washed with PBS and resuspended in the benzidine solution for 5 minutes. Then the percentage of benzidine positive K562 cells was determined using a light microscope with at least 500 cells counted each time.

## 2.11 Giemsa staining of blood smear of circulating blood from E10.5 embryos

To analyze the morphology of primitive red blood cells, intact E10.5 embryos were dissected out of the yolk sac and each was transferred into a 35mm dish with 3 ml PBS. Yolk sacs were opened with tweezers and the embryos were transferred to a fresh dish of PBS containing 12.5 ug/ml Heparin. The umbilical vessels were severed near the embryo's body wall. A pipette was used to aspirate and prevent both the clotting at the exit site and clotting of blood cells in the medium. Once the embryo stopped bleeding, medium was collected and centrifuged. The primitive blood cells were then resuspended in 3ul PBS for making blood smears on a microscope slide. The slide was fixed for 60 seconds in methanol and submerged in 1 ml of Wright Giemsa Stain (Fisher Scientific Company) for 1 to 2 minutes. 1.5 ml of pH6.8 Diluted Buffer (made by mixing pH6.8 Buffer (Fisher Scientific Company) 30 ml with 100 ml of deionized water) was added to the stain-covered slide. The slide was gently rocked for 1 minute to mix the stain and buffer together. The slide stayed 2 more minutes in the mixture. After that, the slide was rinsed with deionized water and allowed to air dry. Slides were viewed using a NIKON ECLIPSE 55i microscope with NIKON Plan 40X objective lens.

## 2.12 Cell cycle and apoptotic study

Single cell suspensions were made from E10.5 yolk sacs, and were cultured in 3334 medium (StemCell Technology) at 37°C for 3 days for apoptosis analysis and for cell cycle studies. Briefly, methylcellulose medium in each dish was dissolved thoroughly in 1X PBS 10 ml. The medium was centrifuged at 1500 rpm for 7 minutes. The cells were resuspended in 1 ml cold PBS and readied for use. For apoptosis analysis,  $1 \times 10^5$  cells from each sample were stained with annexin V-phycoerythrin (BD Biosciences) and Topro 3 (Molecular Probes), following the instructions of the manufacturer. For cell

cycle studies, the cells were stained with propidium iodide. All analyses were performed by flow cytometry with the use of the BD FACSCalibur Flow Cytometer (BD Biosciences).

### 2.13 K562 cell hemin induction

K562 cells (empty vector control and Dot1L KD) were maintained in RPMI complete medium (1% sodium pyruvate, 1% non-essential amino acid, 1% L-glutamine, 10% FBS, and 1% penicillin/streptomycin) containing puromycin 1ug/ml, hemin 50uM at concentration of  $500 \times 10^3$  cells/ml in a 24-well plate. Cells were cultured at 37°C for 36-48 hours. Then cells were centrifuged and supernatant was discarded. Cells were washed 2 times with RPMI complete medium to get rid of exogenous hemin. Finally cells were resuspended in 80ul complete medium and ready for benzidine staining analysis.

### 2.14 *Dot1L* knock down in K562 cells

Lentiviral vectors pGIPz and pLKO.1 containing shRNA targeted to *hDot1L* as well as empty control vectors (Open Biosystems) were amplified in LB medium with 100ug/ml Ampicillin. Minipreps (Qiagen) were performed to extract sufficient amount of plasmids for K562 cell transfection. Briefly, pelleted bacterial cells were resuspended in 250 ul Buffer P1 and transferred to a microcentrifuge tube. 250 ul Buffer P2 was added and the tube was inverted 4-6 times to mix well. 350 ul Buffer N3 was added and the tube was inverted 4-6 times immediately, but gently. The tube was centrifuged for 10 minutes at 13,000 rpm (~17,900xg) in a table-top microcentrifuge. The supernatant in the tube was poured or pipetted into the QIAprep spin column. The column was centrifuged for 30-60 seconds and flow-through was discarded. 0.75 ml Buffer PE was added to wash the column and centrifuge for 30-60 seconds. Flow-through was discarded and centrifuge for

an additional 1 minute to remove residual wash buffer. Then the QIAprep column was placed in clean 1.5 microcentrifuge tube. To elute DNA, 50 ul Buffer EB was added to the center of the QIAprep spin column, let it stand for 1 minute, and centrifuge for 1 minute. DNA concentration was determined by NanoDrop ND 1000 spectrophotometer (Thermo Scientific). Transfection of K562 cells with lentiviral vectors was performed per the following protocol (for each sample):

- 1) resuspend  $1 \times 10^6$  cells in 1 ml Opti-MEM® (Invitrogen);
- 2) mix 1.6 ug plasmid DNA with 100ul Opti-MEM®;
- 3) mix Lipofectamin (Invitrogen) 4ul and 96ul Opti-MEM®. Stay at room temperature for 5 minutes;
- 4) mix solutions from step 2) and 3). Stay at room temperature for 20 minutes;
- 5) add mixture from step 4) to the 1 ml cells in Opti-MEM® in one well of a 6-well plate. After culture cells in 37°C incubator for 5 hours, 1ml RPMI complete medium was added;
- 6) next day, puromycin was added at concentration of 1ug/ml.

After one week, the majority of transfected K562 cells were puromycin-resistant. And pGIPz vector transfected cells were more than 90% GFP positive. Certain amount of cells was used to perform real-time PCR to test Dot1L knock down efficiency.

## 2.15 Lentiviral transduction of G1E cells

Lentivirus was produced by transfection of HEK293T cells with shRNA plasmid (pGIPz) targeted to *mDot1L* (Open Biosystems). The day before transfection (day0), plate the HEK293T cells at a density of  $5.5 \times 10^6$  per 100 mm plate in complete medium (DMEM supplemented with 5% heat inactivated FBS, 100units/ml penicillin and



100ug/ml streptomycin). On the day of transfection, 37.5ug DNA was diluted into 1ml of serum-free medium in a 1.5ml tube. 187.5ul of Arrest-In (Open Biosystems) was diluted into 1ml of serum-free medium in a separate 1.5ml tube. The diluted DNA and the diluted Arrest-In reagent in the two tubes were mixed rapidly to form the transfection complexes and incubated for 20 minutes at room temperature. The complete growth medium was aspirated from the HEK293T cells. The transfection complexes and an additional 3ml of serum free medium (total 5ml) were applied onto the cells. The plate was returned to 37°C incubator with 5% CO<sub>2</sub> for 3-6 hours. Then the transfection mixture was aspirated and 12ml standard culture medium was applied. The cells were returned to 37°C incubator with 5% CO<sub>2</sub>. After 48-72 hours of incubation, GFP expression was examined microscopically, which is an indication of transfection efficiency. Virus-containing supernatant was harvested 48-72 hours post-transfection by removing medium to a 15ml sterile, capped conical tube. For each transduction, 1X10<sup>6</sup> G1E cells were resuspended in 2ml virus-containing medium in one well of a 6-well plate supplemented with 15% FBS, 50ng/ml SCF, 2U/ml EPO. Puromycin was added to concentration of 1ug/ml after cells were cultured in 37°C incubator for 24 hours. GFP positive cells were monitored as an indication of transduction efficiency.

## 2.16 Bone marrow and liver cell extraction from tissue

Bone marrow cells (*Trpc6* highly expressed) were isolated from mouse femurs. Mice were sacrificed using CO<sub>2</sub> and cervical dislocation and was saturated with 70% alcohol. The skin was cut and removed from the lower part of the body. Remaining muscles from the pelvic and femoral bones were cleaned. Each end of the bone was cut and a 25g needle and 5ml syringe filled with cold PBS were used to expel bone marrow

from both ends of the bone into a 50ml Falcon tube. Then a 27g needle attached to a 5ml syringe was used to aspirate and expel the bone marrow cells until the cell aggregates were broken up. For liver cell isolation, mouse liver was dissected into cold PBS. The whole liver tissue was minced into small pieces. The tissue pieces were ground between two pieces of microscope slides in PBS containing protein inhibitor cocktail (Sigma, P8340), until no big chunks of tissue were visible. The suspension was passed through a 70um nylon strainer (BD Biosciences) to obtain single cells. The bone marrow cells and liver cells were then ready for subsequent experiments.

## 2.17 Western Blot analyses

Western blot analyses of the extracted histones were performed with enhanced chemiluminescence following standard protocols. Antibodies against mono-methyl (ab2886), di methyl (ab3594), and tri-methyl H3K79 (ab2621) were obtained from Abcam Inc. Antibodies against unmodified histone H3 and methylated histone H3 at lysines 4, 9, 27, and 36 were all obtained from Cell Signaling Technologies.

Western blot analyses of TRPC6 from different tissues were performed following standard protocols. Cells from yolk sac, bone marrow or liver were lysed in Nonidet-P40 buffer on ice for 30 minutes. The lysates was centrifuged at 12000 rpm at 4°C for 20 minutes. Supernatant was collected and protein concentration was measured using Pierce<sup>®</sup> BCA protein assay kit (Thermo Scientific). Protein was mixed with NuPAGE LDS 4X sample buffer (Life Technologies) and heated at 95°C for 5 minutes. Protein samples were loaded onto a 10%, 30ul BioRad precast gel (Bio-Rad) and run at 100V for 45 minutes. PVDF (positively charged nylon) was soaked in Methanol for 1 minute and then transferred to cold transfer buffer for 3-5 minutes. Gels were taken out and

equilibrated in transfer buffer for 5 minutes. A transfer sandwich was created on Saran wrap and put into the transfer apparatus for transfer at 400mA for 4 hours. After transfer, the PVDF membrane was blocked in 5% BSA for 30-45 minutes. The membrane was then incubated with 1:500 mouse anti-TRPC6 primary antibody (Abcam, mAbcam63038) or 1:2000 mouse anti beta actin primary antibody (Abcam, mAbcam 8224) for 4 hours at 4°C with shaking. Then the membrane was washed 5 times with TBST buffer at 4°C with shaking. The membrane was incubated with 1:5000 secondary antibody (Goat anti mouse IRDye CW800) for 45 minutes at 20°C. The membrane was washed another 5 times with TBST. The image was developed with Odyssey CLx infrared image system (LI-COR Biosciences) following the instructions of the manufacturer.

## 2.18 Chromatin immunoprecipitation (ChIP) assay

G1E cells, bone marrow cells or liver cells were used in my study. For each assay,  $10 \times 10^6$  cells were resuspended in 10ml IMDM medium containing 5% FBS. 270ul 37% Formaldehyde was added (final concentration 1%) and the cells were incubated at 37°C for 10 minutes. 540ul 2.5M glycine was added (final concentration 0.125M) and the cells were incubated at room temperature with rotation for 10 minutes. The cells were washed twice with PBS and transferred to a new 15ml tube. 500ul SDS lysis buffer with protease inhibitor was added and the cells were kept on ice for 10 minutes. The lysate was sonicated with Branson Sonifier 250 Analog Ultrasonic Homogenizer (Branson Ultrasonics) 15 seconds for 4-6 times to reduce DNA length to between 200 and 1000bp. The lysate was centrifuged at maximum speed at 4°C for 10 minutes. Clear supernatant was removed to another 1.5ml tube (can be stored at -80°C until use). To reduce nonspecific background, the chromatin solution (about 500ul) was precleared with 75ul

Protein A Agarose/Salmon Sperm DNA and incubated at 4°C with rotation over night. The solution was centrifuged 3000rpm for 5 minutes. The supernatant was transferred to a new tube and diluted 10 fold in ChIP Dilution Buffer. 1ml of the chromatin solution was used as “input”. Antibodies for H3K79me, H3K79me2, and H3K79me3 were purchased from Abcam Inc. Normal rabbit serum (Biosource international) was used as negative control. For each reaction, 2ug antibody was added to 1ml of chromatin solution and was incubated at 4°C with rotation for 4 hours. The chromatin solution was mixed with 65ul Protein A Agarose/Salmon Sperm DNA and incubated at 4°C with rotation for 2 hours. The solution was centrifuged at 3000rpm for 5 minutes at 4°C and supernatant was discarded. The Protein A agarose beads were washed for 3-5 minutes on a rotating platform sequentially with 1ml of each of the buffers listed below:

Low Salt Immune Complex Wash Buffer

High Salt Immune Complex Wash Buffer

LiCl Immune Complex Wash Buffer

1XTE

1XTE

250ul elution buffer was added to the beads. The beads were vortexed briefly and were incubated at room temperature for 15 minutes with rotation. The beads in elution buffer were centrifuged and supernatant was carefully transferred to another tube. The elution process was repeated and elutes were combined. 20ul 5M NaCl was added to the combined eluates and crosslinks were reversed at 65°C for 4 hours. 10ul 0.5M EDTA, 20ul 1M Tris-HCl, pH6.5, and 2ul 10mg/ml Proteinase K were then added to the eluate mix and incubated at 45°C for 1 hour. The DNA was recovered by phenol/chloroform

extraction and ethanol precipitation. Real-time PCR was performed to quantify precipitated DNA with the use of the Applied Biosystems 7500 real-time PCR system. Primer pairs for analyzing the *Trpc6* locus, *Gata-2* locus, and *pu.1* locus were designed with MacVector software (MacVector Inc.) and are listed below:

**Primer sequences for *Trpc6* locus**

*Trpc6* promoter1:

5'-AAG CAG GGC TCA CTG AAT CTG G-3'

5'-GGC ATT TTC CGA TGG TGT CTG-3'

*Trpc6* promoter2:

5'-CCC AAA TAA AGA ATG TGC CTG G-3'

5'-CGC TGA AGA GTT ACT ATG TCA ACC G-3'

*Trpc6* TSS:

5'-GAG AGC CAG GAC TAT TTG CTG ATG-3'

5'-TGC CCT CGC CCA TAC TTA CAA G-3'

*Trpc6* Middle:

5'-TCT ACC TCC TGA TGC TGG GCT TAC-3'

5'-GGG GTT TGA AGA GAT GAG AGT GC-3'

*Trpc6* End:

5'-TGC CCT ACA AAG CAA TGA AAG G-3'

5'-AAA GAG AGC GTG AGC CCA ACA C-3'

**Primer sequences for *Gata-2* locus:**

1S promoter:

5'- GCATAACGGTGTGTCTGAAAGGAG -3'

5'- GGATGTAATCTCTGAAAGCCTGCG -3'

1G promoter:

5'- TTCCCTCTCCCTAAAGACCGTC -3'

5'- TAGTAGACCACCAGGCTGAACTGC -3'

1S exon:

5'- CGGAGTCGGAATCTTTTCAGTGG -3'

5'- CGAATGGTTCAATGCCCAGC -3'

1G exon:

5'- TCTTATGCTTTGGGGAGAGGTTG -3'

5'- ACACGGATAGGTCCTGACATCG -3'

Intron4:

5'-GACATCTGCAGCCGGTAGATAAG

5'-CATTATTTGCAGAGTGGAGGGTATTAG'

**Primer sequences for *pu.1* locus:**

5'URE:

5'- GCC CAG GCT AGG GAA GTT TG -3'

5'- GAG AGC AGA GCA CTT CAT GGC TA -3'

3'URE:

5'- GGG AGG CAG AGC ACA CAT G -3'

5'- GTT TCC ACA TCG GCA GCA G -3'

Promoter:

5'- GTA GCG CAA GAG ATT TAT GCA AAC -3'

5'- GCA CAA GTT CCT GAT TTT ATC GAA -3'

## 2.19 Flow cytometric analysis and fluorescence activated cell sorting

In order to analyze the progenitor population in yolk sac cells, single cell suspensions from yolk sac were incubated with anti-c-Kit antibody conjugated to phycoerythrin cyanine 5.5 (eBioscience Inc) and anti-CD41 antibody conjugated with phycoerythrin (eBioscience Inc) at 4°C for 30 minutes. The cells were washed with FACS buffer twice and resuspended in 200ul FACS buffer for flow cytometry analysis using BD Accuri<sup>®</sup> C6 Flow Cytometer. In some experiments, the progenitor population among yolk sac cells were analyzed by staining with anti-c-kit antibody and anti-CD45 antibody conjugated with fluorescein isothiocyanate (BD Pharmingen).

In order to obtain the enriched progenitor cells from yolk sac, single cell suspensions from yolk sacs were incubated for 30 minutes at 4°C with anti-c-kit antibody conjugated with phycoerythrin cyanine 5.5 (eBioscience Inc). The c-kit positive cells were isolated by cell sorting with the use of a BD FACSAria cell sorter (BD Biosciencys). Sorted cells were either cultured for colony-forming unit assays or used for gene expression studies.

## 2.20 Measure calcium concentration changes in yolk sac cells in response to EPO

A fluorescence microscopy-coupled digital imaging system was used to measure calcium concentration change of whole yolk sac cells when the cells were treated with EPO. Fura2 (Invitrogen) was used as the detection fluorophore. 50mm culture dishes (BD Biosciences) were coated with poly-lysine (Sigma) for 10 minutes at room temperature and then air-dried. After the yolk sacs were dissociated into single cells, the cells were washed with PBS 3 times to completely remove FBS. The cells were resuspended in 40ul

RPMI 1640 (Sigma) without FBS and were loaded into the center of a culture dish coated with poly-L-lysine. The cells were fixed for 10 minutes at room temperature without shaking or moving. The medium was aspirated carefully without touching yolk sac cells attached to the bottom. A mixture of 1 $\mu$ M Fura2 and pluronic F127 (Invitrogen) (1:1 in volume) was added on top of the cells and the cells were stained at 37°C for 30 minutes. The staining solution was removed and the cells were covered with 150 $\mu$ l RPMI containing 10% FBS. At the beginning of the experiment, mouse EPO (R&D systems) was added at a final concentration of 10unit/ml. The sample was analyzed immediately with the confocal fluorescence microscopy on a Nikon TE2000U microscope. Fura2-loaded cells were visualized with digital video imaging and fluorescence was quantified using the fluorescence intensity ratio of the emission (510nm) measured following excitation at 340nm divided by the emission following excitation at 380nm. Calcium responses from cells in each sample were measured for 20 minutes. Finally, the data was calculated and analyzed by Metamorph6.1 (Universal Imaging Corp., Downingtown, PA).



## Chapter 3

### **Early mammalian erythropoiesis requires the DOT1L methyltransferase**

#### 3.1 Introduction

Among the first differentiated cell types to emerge in the developing mammalian embryo are the blood cells. In the mouse, the process of blood development, hematopoiesis, begins at approximately embryonic day 7.0 to 7.5 (E7.0-E7.5), when cells originating at the primitive streak migrate to the site of yolk sac formation.<sup>120</sup> By E7.5, the cells coalesce into blood islands, where they mature, proliferate, and differentiate<sup>22</sup>. These early hematopoietic progenitors, termed primitive erythroid colony-forming cells, are nucleated red cells, which express primitive globins and can carry oxygen to nourish the developing embryo on the initiation of blood flow after E8.5<sup>28,120-122</sup>. The presence of these primitive progenitors is transient, peaking in numbers at E8.0 and disappearing by E9.0, whereas the progeny erythrocytes persist throughout gestation<sup>24</sup>. After E8.5, a second wave of hematopoietic progenitors emerges from a variety of sites, including the vasculature about the aorta-gonad-mesonephros and the yolk sac. These cells enter the circulation and migrate to the developing fetal liver. There, they proliferate and undergo “definitive” maturation, giving rise to multiple adult hematopoietic lineages, including mature, enucleated erythrocytes<sup>1</sup>. This multi-step process of hematopoiesis and the fate decisions of the developing cells are regulated by the precisely controlled, sequential induction and silencing of gene expression in response to a variety of growth and differentiation factors<sup>5</sup>. The identity of the cell-type specific genes that direct

differentiation, the factors controlling their expression, and the mechanisms by which their expression is regulated are currently active areas of investigation.

Although many mechanisms probably contribute to the coordination of gene expression during hematopoiesis, it is becoming increasingly clear that epigenetic change is an important strategy used by eukaryotes to accomplish such complex regulation<sup>123-125</sup>. One of the most widely studied regulators of epigenetic change is the enzymatic modification of histones. In eukaryotic cells, histones and histone-associated proteins along with DNA are packaged into nucleosomes, which are the fundamental repeating structural units of chromatin<sup>126</sup>. The histones can be posttranslationally modified in a variety of ways, including acetylation, phosphorylation, ubiquitination, and methylation. These modifications influence chromatin structure, facilitate interactions between nucleosomes, recruit non-histone proteins to the DNA, and can potentially regulate transcription<sup>10</sup>.

Histone methylation has been shown to play an important role in regulating both chromatin function and gene expression. Most histone lysine methyltransferase (HLMT) family members contain a conserved SET domain, which is required for enzymatic activity and believed to be a signature motif for this class of enzymes<sup>10,104,127</sup>. One member of this family that is an exception to this rule is disruptor of telomere silencing 1 (DOT1), an HLMT first identified in *Saccharomyces cerevisiae*<sup>97</sup>. Unlike other HLMT, DOT1, as well as its mammalian homologue DOT1-like (DOT1L), lacks a SET domain<sup>97,126</sup>. Further, the substrate residue for DOT1/DOT1L is lysine 79 of histone H3 (H3K79), which is not located on the N-terminal tail of H3, but rather is located within a loop in the globular domain of the histone that is exposed on the nucleosome surface<sup>89</sup>.

These unique aspects of Dot1L may underlie the distinctive biologic roles that have been identified for this enzyme relative to other HLMTs<sup>11,126,128</sup>. The consequences of H3K79 methylation are incompletely understood, but this histone modification has been shown to be strongly associated with actively transcribed chromatin in several mammalian cell lines<sup>13</sup>.

A mouse line was established by another group, in which DOT1L activity was disrupted by targeted mutation. This study focused on the embryonic development of *Dot1L* knockout (KO) mice, DOT1L function in embryonic stem (ES) cell growth, and aberrant telomere elongation in *Dot1L* KO ES cells<sup>12</sup>. Defects in yolk sac angiogenesis were reported, but no alterations in hematopoiesis were described. In other studies, however, H3K79 methylation was observed in the  $\beta$ -globin locus, suggesting a possible role for this histone modification in erythropoiesis<sup>129,130</sup>. To date, no detailed molecular level studies of the role of DOT1L in hematopoiesis have been carried out.

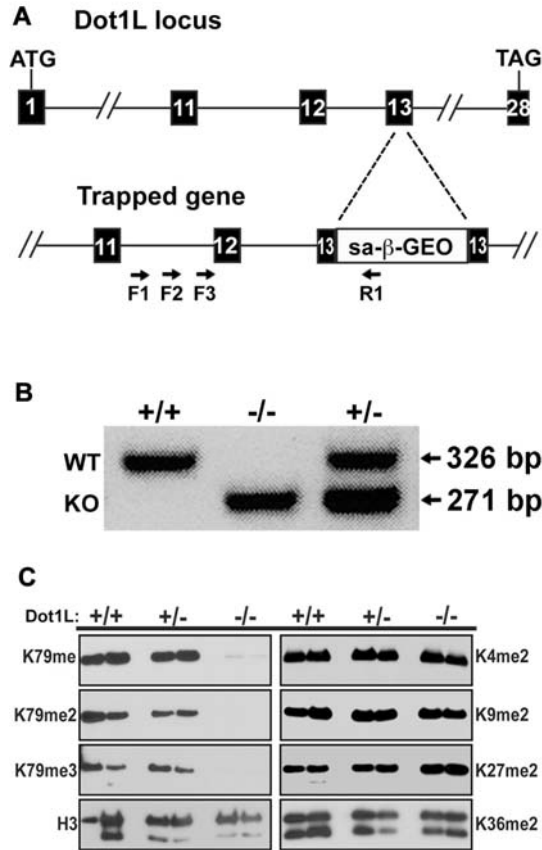
A mouse line in which a gene trap has been used to abrogate DOT1L activity was created in our laboratory<sup>7</sup>. After analysis of the mouse line, we confirmed that DOT1L function is essential for embryogenesis, with embryonic lethality occurring at mid-gestation in *Dot1L* deficient mice. Notably, we observed a defect in early erythropoiesis as a primary contributor to this embryonic lethality. This work highlights the importance of H3K79 methylation in embryogenesis and has begun to shed light on DOT1L function in embryonic hematopoiesis.

## 3.2 Results

### 3.2.1 Generation of mouse lines containing mutant allele of *Dot1L*

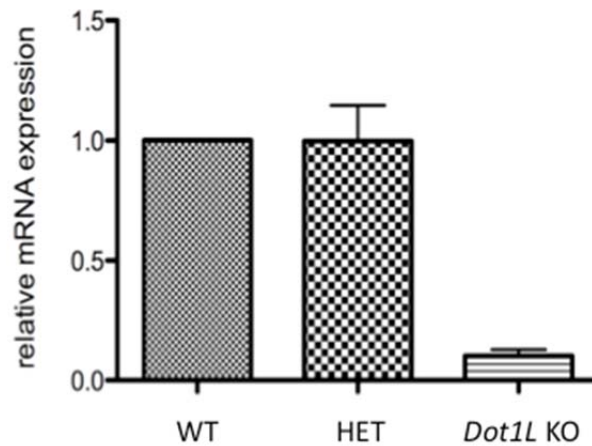
To generate an *in vivo* model of deficient Dot1L function in the mouse, an ES cell line was obtained from Bay Genomics with an insertion mutation in the *Dot1L* locus. Four primers were designed to determine the exact position of the gene trap insertion. Three forward primers were within intron 11 of the *Dot1L* gene (F1, F2, F3), whereas the reverse primer was within the inserted gene trap (R1) (Figure 3.1 A). Sequence analysis identified the gene trap cassette within exon 13 after the 26<sup>th</sup> nucleotide. This position is 5' to the region encoding the critical nucleosome-binding region of DOT1L (amino acids 390-407), which is essential for DOT1L enzymatic activity<sup>13</sup>. Thus, insertion in this area would be predicted to result in a substantial disruption of DOT1L function. Indeed, this gene trap has been used recently to analyze DOT1L loss of function in fibroblasts<sup>13</sup>. Embryos were generated with gene-trapped stem cells, and genotyping PCR primers were used to distinguish wild-type (WT), *Dot1L* heterozygous mutant, and KO mice and embryos (Figure 3.1 B).

To assess the functional consequences of DOT1L loss, MEF were derived from WT, heterozygous, and homozygous mutant (KO) E10.5 embryos. In MEF from *Dot1L*-KO embryos, there was no significant expression of *Dot1L* mRNA (Figure 3.2). Because antibodies were not available to measure protein expression, DOT1L activity was assessed by analyzing H3K79 methylation. Histones were prepared from each genotype, and immunoblot analysis showed that mono-, di-, and tri-methylation of H3K79, the reported substrate for DOT1L<sup>89</sup>, were completely abrogated in the KO MEF, whereas in both WT and heterozygous MEF, H3K79 methylation was unaffected (Figure 3.1 C). The patterns of H3K79 methylation were not influenced by either presence or absence of serum (Figure 3.1 C, compare left with right panels), conditions known to influence



**Figure 3.1. Generation of mice with mutant *Dot1L* alleles.** (A) Schematic representation of the “gene trap” construct used to generate the mutant *Dot1L* alleles (KO). The numbered boxes represent exons of the *Dot1L* gene. Arrows represent PCR primers used to amplify DNA from ES cells to identify the exact point of insertion of the gene trap. (B) PCR genotyping using DNA from embryos generated by interbreeding heterozygous *Dot1L* mutant mice. PCR primers were generated that distinguished WT and KO alleles. As expected, both alleles were detected in heterozygous embryos (lane 3). (C) Absence of H3K79 methylation in MEF from *Dot1L*<sup>-/-</sup> embryos. MEF were

derived from E10.5 embryos and either serum-starved for 24 hrs. (left bands) or treated overnight with 20% FCS (right bands). Histones were acid-extracted from cells and proteins were resolved by SDS-PAGE. Blots were probed with antibodies against histone H3 Lysine 79 mono- (H3K79me), di- (H3K79me2), and tri-methylation (H3K79me3). Other antibodies against Histone H3 modifications were used to probe the blot including: unmodified Histone H3 (H3), dimethylated lysine 4 (H3K4me2), dimethylated lysine 9 (H3K9me2), dimethylated lysine 27 (H3K27me2), and lysine 36 (H3K36me2).



**Figure 3.2 *Dot1L* expression levels in WT, heterozygous, and *Dot1L* KO cells:**

RNA was extracted from WT, HET, and *Dot1L* KO mouse embryonic fibroblast cells (MEFs), converted into cDNA, and *Dot1L* expression was measured by Real-Time PCR. Average value for WT cells was normalized to 1 and HET and KO values were calculated. Error bars represent standard deviation of calculated values from five (WT), four (Het), and 14 (KO) independent cell lines.

Primer sequences:

Forward: GGC CCA AAA GGA AGA AAA TCC

Reverse: TCA GGT CAT TGT AGG TCA GC

**Table 3.1 survival rate at different stages**

	E10.5	E11.5	E13.5	P21
Total embryos	227	40	18	151
<i>Dot1L</i> <sup>+/+</sup> (WT)	61 (27%)	14 (35%)	4 (22%)	53 (35%)
<i>Dot1L</i> <sup>+/-</sup> (Het)	114 (50%)	19 (48%)	12 (67%)	98 (65%)
<i>Dot1L</i> <sup>-/-</sup> (KO)	52 (23%)	7 (18%)	0 (2)* (11%)	0 (0%)

\*Embryos were dead but not resorbed.

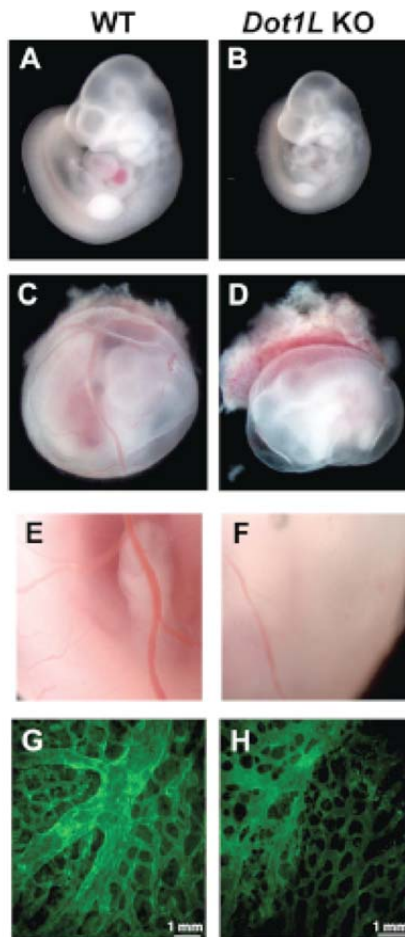
histone methylation. Other H3 lysine methylation marks, including di-methylation of K4, K9, K27, and K36, were unaffected in mutant MEF (Figure 3.1 C). These results confirm that DOT1L is specific for H3K79 and is the only detectable H3K79 methyltransferase during embryogenesis, consistent with previous reports<sup>12,89</sup>.

### 3.2.2 *Dot1L* deficiency results in embryonic lethality and severe anemia

The *Dot1L* heterozygous mice appear phenotypically normal and fertile. However, on examination of more than 150 pups, no viable *Dot1L* KO offspring were produced after breeding heterozygotes (Table 3.1), indicating that *Dot1L* deficiency resulted in embryonic lethality. To identify the timing and cause of the *Dot1L* KO-dependent lethality, WT, heterozygous, and KO embryos were examined at several time points during development. The genotype distribution at E9.5 occurred at expected Mendelian ratios, and there were no distinguishable phenotypic differences between WT, heterozygous, and KO embryos harvested from the litters at this time point. At E10.5, the distribution of viable embryos was close to the expected Mendelian ratios, but most KO embryos were reduced in size compared with WT littermates (Figure 3.3 A-B). In addition, *Dot1L* KO embryos exhibited a striking near absence of red blood cells, which resulted in an overall paler appearance than the WT embryos (Figure 3.3 C-D).

Examination of the yolk sacs showed that those from KO embryos also contained less blood and exhibited altered vessels in comparison to the yolk sacs from WT embryos (Figure 3.3 E-F). Immunofluorescence analysis of WT and KO yolk sacs with the use of anti-platelet endothelial cell adhesion molecule (CD31) showed that both contained abundant endothelial cells, which constitute the complex vascular plexus network present at this time. In WT yolk sacs at E10.5, progression of angiogenic remodeling was

**Figure 3.3 Defects in hematopoiesis and vascular development in *Dot1L* KO embryos.**



(A-B) Representative pictures of WT (A) and *Dot1L* KO (B) embryos at E10.5, showing reduced embryo size as well as significant anemia in the *Dot1L* knockout embryos. Note the overall pale appearance of the knockout embryo. (C-D) Representative pictures of whole mount WT (C) and *Dot1L* KO (D) embryos with surrounding yolk sac show that yolk sacs from *Dot1L* KO contain less blood and exhibit a reduction in the number of vessels compared to those from WT. A-D were viewed using a NIKON SMZ800 stereo microscope with Plan 1X objective lenses (total 10X) and images were taken with a LEICA DFC480 camera using Leica Firecam software at room temperature. (E-F) Representative enlarged pictures (20X) reveal that blood vessels in KO yolk sacs exhibit abnormal caliber and contain less blood compared to WT. (G-H) Representative immunofluorescence confocal images of E10.5 yolk sacs stained with anti-CD31 show a mild defect in vascular remodeling in the *Dot1L* KO embryos (H). Pictures are representative of 7-10 individual embryos. Pictures were viewed with an Olympus 1X71 microscope with 10X UPLANF1 objective lens and images were taken by Olympus DP71 camera using Olympus DP71 controller software. The fluorochrome was FITC.

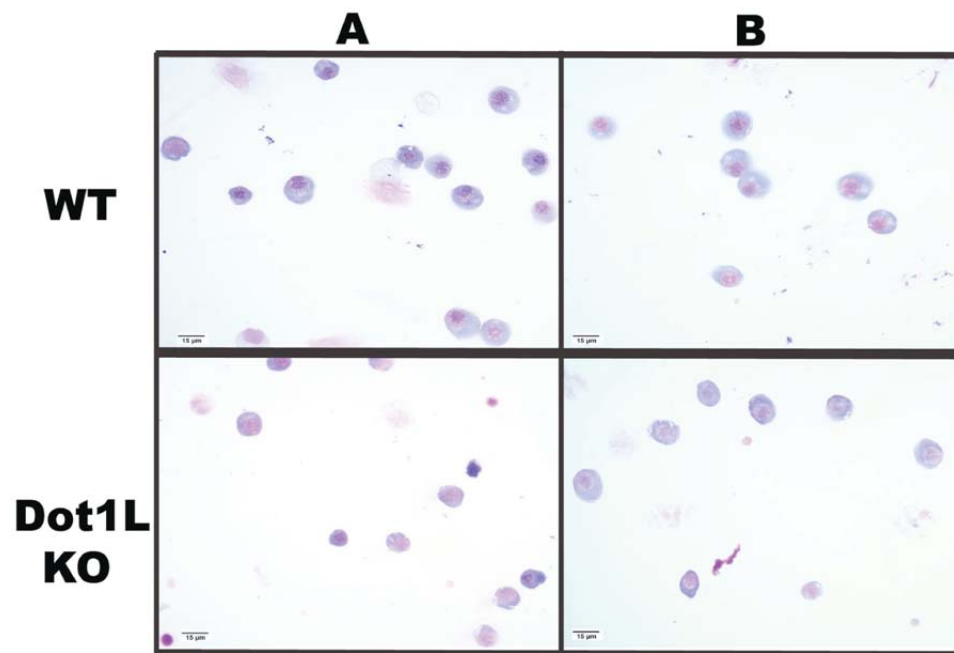


observed, as indicated by the large caliber vessels of the vitelline artery and vein. In *Dot1L* KO yolk sacs, the remodeling process was present; however, the vessels appeared disorganized and exhibited relatively deficient vascular branching, similar to the *Dot1L*-deficient yolk sacs described by Jones, *et al*<sup>12</sup>. (Figure 3.3 G-H)

At E11.5, the percentage of viable *Dot1L* KO embryos was much lower than the expected Mendelian ratio, and no KO embryos survived at or beyond E13.5 (Table 3.1). Collectively, these data show that the *Dot1L* KO embryos do not survive past mid-gestation, a phenomenon probably due to the severe anemia and associated defects in vascular remodeling.

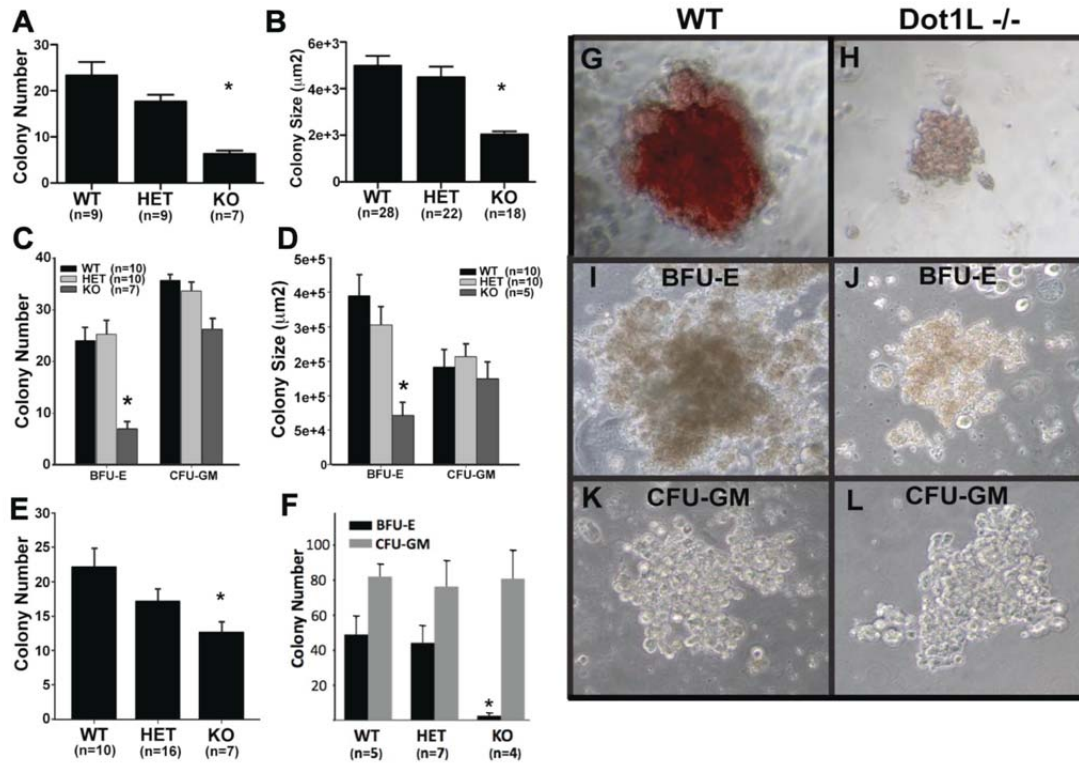
### 3.2.3 DOT1L controls yolk sac erythropoiesis

The severe anemia observed in the KO embryos suggested that DOT1L is essential for hematopoiesis. Because it is thought that a primitive erythroid defect would contribute to anemia observed before E13<sup>1,26</sup>, we first investigated whether the loss of DOT1L affects the formation of primitive erythroid progenitor cells. At E10.5, most circulating, differentiated erythroid cells should be derived from primitive erythropoiesis<sup>1</sup>. A non-quantitative analysis of blood smears at this time point showed normal appearing, nucleated erythroid cells in the *Dot1L* KO embryos of various stages of maturation (Figure 3.4). We carried out more quantitative CFU assays to determine the ability of primitive progenitors to proliferate and form colonies in response to growth factors. With the use of E8.5 embryos and yolk sacs as a source of primitive erythroid progenitors, we found that the *Dot1L* KO cells gave rise to significantly fewer colonies when we applied equal numbers of the cells onto each culture dish (Figure 3.5 A). In addition, with the use of image analysis, we observed that *Dot1L* KO colonies were only



**Figure 3.4 Blood smears of circulating blood from E10.5 embryos:**

Circulating blood from E10.5 embryos was collected in capillary tubes and smears were visualized by Giemsa staining. Pictures were viewed using a NIKON ECLIPSE 55i microscope with NIKON Plan 40X, numerical aperture 0.17 objective lens and were taken with a LEICA DFC480 camera equipped with Leica Firecam software. No morphological differences are observed. Photos are representative of smears analyzed from 6 WT and 4 KO embryos.

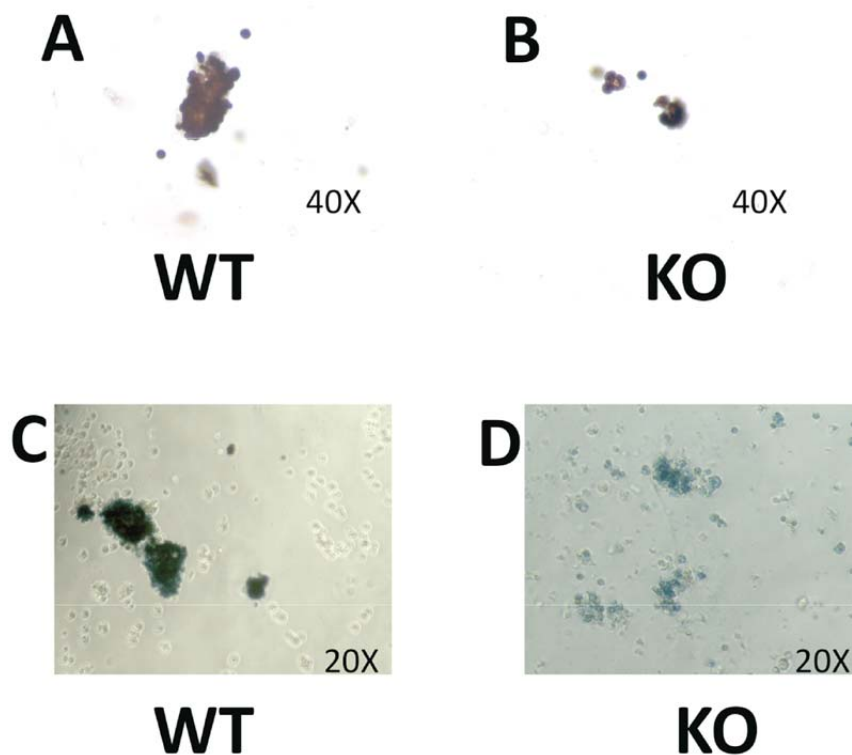


**Figure 3.5 Loss of DOT1L results in the impairment of both primitive and definitive yolk sac erythropoiesis.** (A-B) Identical numbers of dissociated E8.5 yolk sac cells were cultured for 7 days in media specifically supporting the outgrowth of primitive erythroid colonies. Colonies were enumerated (A), and size was measured (B) with ImageJ software. (C-D) Identical numbers of dissociated E10.5 yolk sac cells were cultured in methylcellulose growth medium supporting both erythroid and granulocyte-macrophage progenitors. Colonies were scored and counted (C) after 10-12 days in culture. (D) The area of single BFU-E and CFU-GM colonies was measured to estimate colony size with ImageJ software. (E) Identical numbers of dissociated E10.5 yolk sac cells were plated in methylcellulose medium that only supports the outgrowth of erythroid progenitors (CFU-E and mature BFU-E). Colonies were identified and counted after 3 days of culture. (F) C-kit<sup>+</sup> cells were isolated from individual yolk sacs by sorting. Identical numbers from each genotype were cultured in methylcellulose medium containing erythropoietin for 10 days. Total erythroid colonies were enumerated. (G-L) Representative pictures of primitive erythroid (G-H), BFU-E (I, J), and CFU-GM (K-L) colonies showing size and appearance differences between WT (G, I, K) and Dot1L KO (H, J, L). Figure G-H are derived from experiments described in Figure 3A-3B. Figure I-L are from the experiment described in Figure 3C-3D. Cultures were viewed with a NIKON ECLIPSE TS100 microscope. Images were taken with a LEICA DFC480 camera using Leica Firecam software in room temperature. \*average number (A, C, E) and size (B, D, F) of KO colonies was significantly different from WT and heterozygotes ( $p < 0.01$ ; t-test) Error bars represent standard errors within each group.

approximately one half the average size of WT or heterozygous colonies (Figure 3.5 B, G, H;  $P < .01$ )

We next analyzed definitive hematopoiesis at E10.5, when the anemic phenotype of *Dot1L* KO embryos is obvious, but embryonic viability remains unaffected. At this time, the fetal liver is not yet completely developed, and a substantial reservoir of embryonic hematopoiesis occurs in cells derived from the yolk sac<sup>1,17</sup>. Yolk sacs from E10.5 embryos were dissociated and plated under conditions that support multipotent, erythroid and myeloid progenitor cell differentiation. The number of definitive erythroid progenitors was measured by counting BFU-E colonies, which have a characteristic structure under light microscopy (Figure 3.5 I). The total number of BFU-E colonies obtained from KO yolk sac cells was much lower than the number obtained from WT or heterozygous embryos (Figure 3.5 C). In contrast, the number of CFU-GM was not significantly different among WT, heterozygote, and KO yolk sacs (Figure 3.5 C). The few BFU-E colonies generated from *Dot1L* KO yolk sacs were approximately 3 times smaller than WT colonies, suggesting that *Dot1L*-deficient erythroid progenitors are defective in their ability to optimally proliferate in response to growth factors in the media (Figure 3.5 D, I, J;  $P < .01$ ). No significant difference in the size of the CFU-GM colonies was observed among the different genotypes (Figure 3.5 D, K, L).

We next plated equal numbers of dissociated yolk sac cells on medium that only supports the outgrowth of erythroid CFU colonies (Figure 3.5 E). Under these conditions, significant fewer KO erythroid colonies were derived than from either WT ( $P < 0.01$ ) or heterozygous ( $P < 0.02$ ) cells. The slight difference between WT and heterozygous colony numbers was not statistically significant ( $P = 0.38$ ). Primitive and definitive WT and



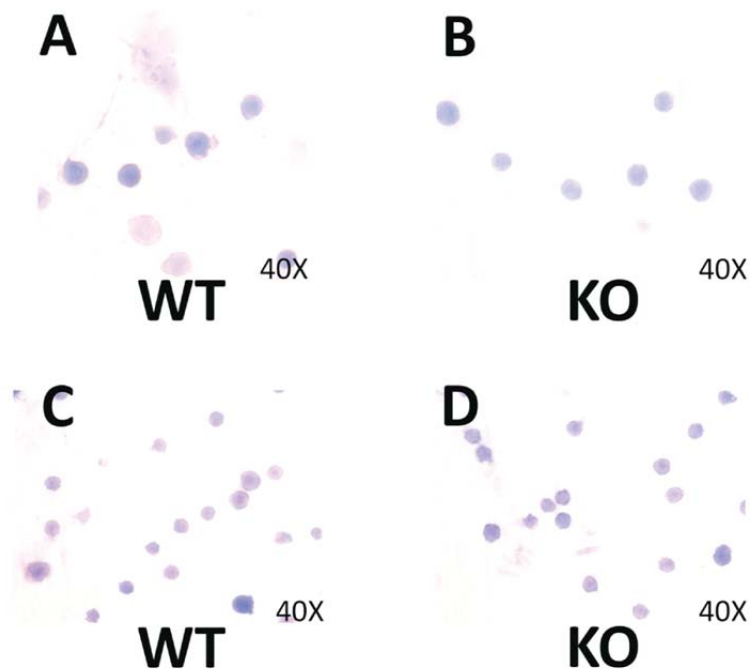
**Figure 3.6 Benzidine staining of primitive and definitive erythroid colonies:**

Benzidine staining was performed on primitive erythroid colonies derived from E8.5 yolk sacs (A, B) and BFU-E colonies derived from E10.5 yolk sacs (C, D). Both WT and *Dot1L* KO showed positive staining, although in WT colonies, the staining was more intense than in *Dot1L* KO cells, due to much greater cell numbers in each colony. Stained colonies were viewed with a NIKON ECLIPSE TS100 microscope equipped with 20X phase contrast, numerical aperture 1.2 objective lens. Images were taken with LEICA DFC480 camera using Firecam software in room temperature. Shown are representative pictures of primitive colonies derived from 4 WT and 3 KO embryos and definitive colonies from 5 WT and 3 KO.

*Dot1L* KO erythroid colonies were all positive for benzidine staining, indicating normal globin production in the KO cells. However, the staining was less intense in KO colonies, because of relatively lower cell numbers in each colony (Figure 3.6). We also collected cells from primitive and definitive erythroid colonies and analyzed them by Giemsa staining. No gross structural differences between WT and *Dot1L* KO were observed at a single-cell level (Figure 3.7).

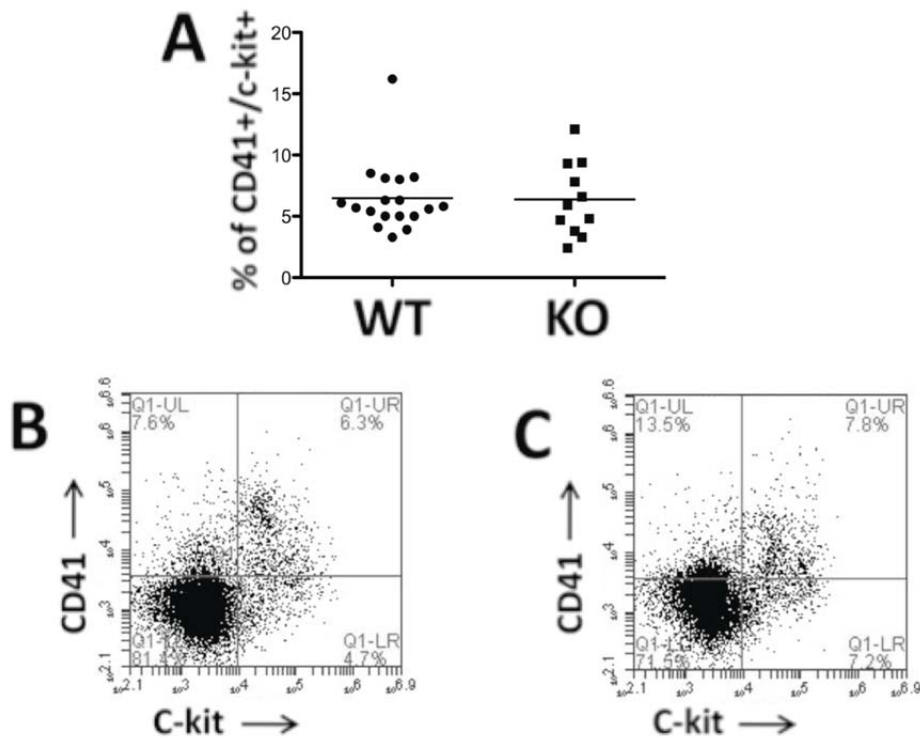
The observed defect in erythropoiesis could be from either a reduction in the relative number of erythroid progenitors or a reduction in the developmental potential of these cells that are present. Hematopoietic progenitors in the yolk sac are enriched within the CD41<sup>+</sup>/c-kit<sup>+</sup> population<sup>19</sup>. Thus, we analyzed CD41<sup>+</sup>/c-kit<sup>+</sup> frequency in E10.5 embryos. No significant differences were observed in the relative percentages of these populations between WT and KO yolk sacs, although the absolute numbers of cells were lower (Figure 3.8). Likewise, we detected no differences in the percentages of total CD45<sup>+</sup>/c-kit<sup>+</sup> cells in yolk sacs (Figure 3.9). Therefore, the definitive progenitors in *Dot1L* KO yolk sacs emerge relatively normally.

C-kit is expressed by most common hematopoietic progenitor cells, including those that give rise to erythrocytes. Identical numbers of sorted, c-kit<sup>+</sup> yolk sac cells from each genotype were cultured under conditions that support both erythroid and myeloid differentiation. After 10 days of culture, no BFU-E colonies developed from KO c-kit<sup>+</sup> yolk sac cells, whereas myeloid colonies (CFU-GM) formed normally (Figure 3.5 F). In contrast, c-kit<sup>+</sup> progenitors from WT and heterozygous tissues formed BFU-E and CFU-GM colonies as expected.



**Figure 3.7 Giemsa staining of cells from WT and KO primitive and definitive erythroid colonies:**

Cells from WT or Dot1L KO primitive (A,B) and definitive (C,D) erythroid colonies were collected in 3 ul PBS, smeared onto microscope slides and visualized by Giemsa staining. Pictures were viewed using a NIKON ECLIPSE 55i microscope with NIKON Plan 40X, numerical aperture 0.17 objective lens and were taken with LEICA DFC480 camera equipped with Leica Firecam software. Pictures are representative stains of 6 WT and 3 KO primitive colonies and 4 WT and 3 KO definitive colonies.



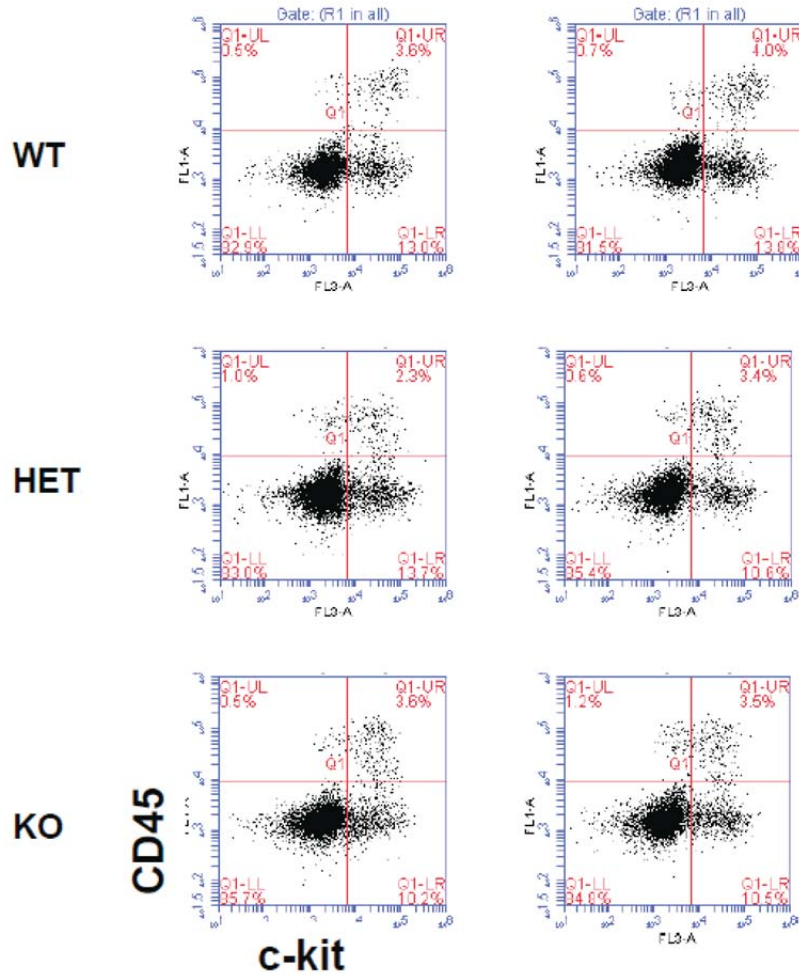
**Figure 3.8 C-kit/CD41 staining of E10.5 yolk sacs from WT and *Dot1L* KO**

**embryos:**

Yolk sacs were collected and dissociated to single cells, and then stained with CD41 and c-kit antibodies. The frequency of double positive cells was similar between WT and *Dot1L* KO yolk sacs (A). Representative figures for WT (B) and *Dot1L* KO (C) are shown.



Genotype	c-kit <sup>pos</sup>	CD45 <sup>pos</sup>	Dble pos.	n
+/+	*16.2 ± 4.1	7.4 ± 2.2	5.7 ± 2.2	5
+/-	12.5 ± 1.9	4.0 ± 0.4	3.0 ± 0.5	4
-/-	*15.4 ± 2.1	6.2 ± 1.0	4.2 ± 0.3	7
*p value	0.85	0.61	0.50	



**Figure 3.9 c-kit<sup>+</sup>/CD45<sup>+</sup> cells in E10.5 yolk sacs:**

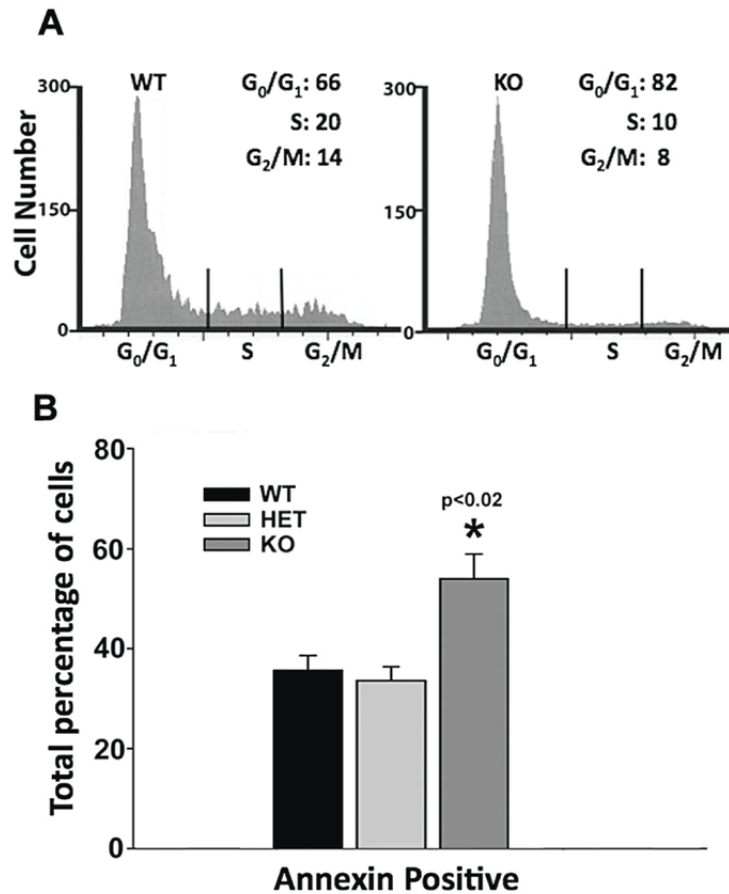
Table showing average percentages of c-kit<sup>+</sup>, CD45<sup>+</sup>, and double positive cells in *Dot1L* WT, HET, and KO yolk sac cells from single embryos. Numbers represent average ± SEM for each genotype.

Representative histograms of wildtype, heterozygous, and homozygous mutant FACS staining of total yolk sac cells for c-kit and CD45.

### 3.2.4 *Dot1L* deficiency blocks cell-cycle progression and promotes apoptosis of hematopoietic progenitor cells

Defective erythropoiesis in the *Dot1L* KO could result from either an alteration in cell-cycle progression or increased apoptotic death of progenitor cells or both. To distinguish between these possibilities, we plated cells from individual E10.5 yolk sacs on erythroid growth media for development into erythroid colonies. After 4 days of growth, we harvested the cells and analyzed their cell-cycle profile by propidium iodide staining and flow cytometry. An analysis of 7 WT and 8 KO embryos showed a significant defect in the KO cells' progression through the cell cycle. We found in WT colonies, an average of 66% of cells were in G<sub>0</sub>/G<sub>1</sub> stages, whereas 20% and 14% of cells were in S and G<sub>2</sub>/M stages, respectively. In sharp contrast, 82% of KO cells were found to be in G<sub>0</sub>/G<sub>1</sub> stages, whereas 10% and 8% of the cells had progressed to S and G<sub>2</sub>/M stages (Figure 3.10 A).

We also examined apoptosis by annexin V staining in E10.5 yolk sac cells. After 3 days of culture in erythroid growth media, *Dot1L* KO cells showed significantly more apoptosis than either WT or heterozygous cells (Figure 3.10 B). These data indicate that DOT1L loss in yolk sac-derived, erythroid progenitors induces both an increased G<sub>0</sub>/G<sub>1</sub> period and an increased susceptibility to apoptosis. We postulate that the requirement for DOT1L in timely cell-cycle progression and survival of the erythroid lineage may be partly responsible for the reduced number and size of erythroblast colonies generated by KO yolk sacs and may contribute to the anemia observed *in vivo*.



**Figure 3.10 Defects in cell cycle progression and increased apoptosis caused by *Dot1L* knockout during erythropoiesis.** (A) Dissociated yolk sac cells of wildtype and *Dot1L* knockout embryos were plated in methylcellulose growth medium supporting erythroid lineage growth and cultured for 4 days. The cells were harvested and cell cycle was analyzed by PI staining and flow cytometry. Differences in percentages of cells in all stages of the cell cycle were significantly different ( $p < 0.02$ ; t-test) (B) Analysis of apoptosis in examined in yolk sac cells cultured in erythropoietin-containing growth medium by Annexin V staining and flow cytometry.

### 3.3 Discussion

Due to the special position of lysine 79 on histone H3 and the unique structure of its methyltransferase—DOT1L, we sought to examine the effects of a germline KO of the *Dot1L* gene on embryonic growth and development. While these studies were ongoing, another group showed that DOT1L loss resulted in embryonic lethality<sup>12</sup>. That group showed that by E10.5, the viability of *Dot1L* KO embryos was the expected Mendelian ratio and that no KO embryos survived beyond this time. The lethality in these embryos was mostly ascribed to defective yolk sac angiogenesis. We observed similar defective yolk sac angiogenesis in *Dot1L* KO embryos. However, because we also observed a selective and profound defect in erythropoiesis, we propose that the vessel-remodeling defects observed in the *Dot1L*-mutant extraembryonic tissues are largely a consequence of altered hematopoiesis and reduced blood flow in these embryos. Indeed, an essential role for blood flow and hemodynamic forces in driving angiogenic remodeling of the yolk sac has been shown previously<sup>122</sup>.

Although *Dot1L* deficiency also resulted in embryonic lethality in our hands, our embryos survived somewhat longer than was reported in study mentioned earlier. In contrast to that study, we found that at E10.5 embryo viability appeared normal. However, most KO embryos were reduced in size compared with WT littermates, and no *Dot1L* KO embryos survived at or beyond E13.5. We believe this discrepancy may be due to the different methods used to generate the mutant embryos. The mutant described by Jones, *et al.* was generated by deletion of exons 5 and 6 of *Dot1L*<sup>12</sup>, whereas the gene trap mutation used in our studies resulted in an interruption of entire 3' of the gene, after exon 13.

Despite the differences in the generation of the KO, both Jones, *et al.* and our findings show that *Dot1L* KO results in the absence of H3K79 mono-, di-, and tri-methylation. Notably, the conclusion of Jones, *et al.*<sup>12</sup> about the absence of mono-methyl H3K79 was based on mass spectrometry rather than immunoblot analysis, which was the basis of our conclusion. This was because the mono-methyl H3K79 antiserum reportedly cross-reacted with histone H3 in their hands. In our studies, this antiserum worked well for Western Blotting, was specific, and had minimal cross-reactivity with histone H3 (Figure 3.1 C). The reason for this difference is not clear; however, it may be related to batch-to-batch variability of this polyclonal rabbit antisera or variability in secondary antibodies or means of detection.

Another group also examined DOT1L loss of function with the use of the same gene trap used in our study<sup>13</sup>. However, we have determined that the gene trap insertion is within exon 13, rather than in intron 12, as was previously suggested<sup>13</sup>. In agreement with our results, that study also reported specificity of the mono-methyl H3K79 antisera without cross-reactivity with H3 by immunoblot. In contrast to our findings, that group concluded that mono-methyl H3K79 was increased in the *Dot1L* KO cells. This conclusion was based on ChIP analyses, which showed enriched mono-methyl H3K79 in certain loci. The reason for this difference is not certain, but it is tempting to speculate that this result could reflect some level of cross-reactivity of the antisera with formalin-fixed H3 in the immunoprecipitations.

Our results clearly show that *Dot1L* deficiency results in a significant defect in erythrocyte development, manifested by the severe anemia observed in E10.5 *Dot1L* KO embryos. At distinct times during development, the yolk sac is a source of progenitor

cells for both primitive and definitive erythropoiesis<sup>1,22</sup>. Our colony forming studies on cells from E8.5 and E10.5 embryos showed that DOT1L loss caused a defect in erythroid progenitors derived from both the primitive and definitive lineages. Analysis of isolated c-kit<sup>+</sup> progenitors at E10.5 indicates that this defect resides in this cell population (Figure 3.5 F). Although the total number of erythroid progenitors was reduced in the KO embryos, the relative percentages of these cells appeared normal (Figure 3.8 and 3.9). This fact, coupled with direct visualization of blood at E10.5 (Figure 3.4), indicates that both primitive and definitive progenitors can be produced and can mature normally. However, their common impaired expansion and survival in colony-forming assays suggests a shared deficiency or inappropriate response to erythroid growth stimuli, resulting in failure to form large colonies *in vitro* and anemia *in vivo*<sup>54,131-133</sup>. This putative altered response to growth factors is probably the primary cause of lethality in these embryos after E10.5. Collectively, these data indicate that DOT1L plays an essential role in the growth and differentiation of the erythroid lineage during early hematopoiesis.

As I discussed in the previous chapter, the primitive and definitive erythrocytes derived from yolk sac could only meet the needs of embryonic growth temporarily. The continuous generation of blood cells from long-term hematopoietic stem cells (HSC) is the ultimate aim of adult hematopoiesis<sup>1</sup>. The first long-term HSC, which have the capacity of engrafting adult recipients, could be identified in the AGM region at E10.5<sup>30</sup>. HSC then migrate through the blood stream and seed the fetal liver at E11. Subsequently, the hematopoietic niche shifts gradually from the fetal liver to the bone marrow cavity and the fetal liver stops functioning as an hematopoietic organ soon after birth<sup>1,16</sup>. Since

our *Dot1L* mutant mice suffer embryonic lethality at mid-gestation, we could not analyze DOT1L function in HSC using our mouse model. Soon after we reported our data, at least two other groups carried out studies on DOT1L function in adult hematopoiesis in mice<sup>15,115</sup>.

To circumvent embryonic lethality, an inducible *Dot1L* KO mouse line was generated. *Dot1L* conditional mice were crossed with a Cre-ER mouse line (R26-Cre-ER), so that *Dot1L* can be deleted *in vivo* by Tamoxifen (TAM) injection. They performed real-time PCR to demonstrate that TAM treatment can induce *Dot1L* depletion efficiently. Only one week after injection, conditional KO mice showed signs of anemia and bleeding, while WT control group appeared normal. Consistent with our observations, a general hypocellularity in the bone marrow of conditional KO mice was observed when compared with WT controls<sup>115</sup>. By doing flow cytometry and bone marrow transplantation experiments, both these two groups proved that DOT1L is critical in maintaining normal adult hematopoiesis and that depletion of DOT1L leads to bone marrow failure<sup>15,115</sup>.

The DOT1L studies in adult mice are very good extensions of our work, and supplement our DOT1L studies in embryonic development. Indeed, both of them demonstrated a critical role for DOT1L in maintaining normal adult hematopoiesis in mice. However, their results are not completely consistent with our research. In our research, the emergence of a progenitor cell population was not significantly affected by *Dot1L* mutation, which was demonstrated by flow cytometric analysis of CD41/c-kit double stained and CD45/c-kit double stained E10.5 yolk sac cells. The percentage of double positive cells was almost the same between WT and the *Dot1L* KO group<sup>7</sup>. This

indicated that the progenitor cells could still emerge relatively normally in absence of DOT1L. But they could not proliferate and differentiate properly, possibly due to defect in their response to erythroid growth stimuli. In contrast, in the conditional *Dot1L* KO studies, they found that the percentage of multiple progenitors including CMP (common myeloid progenitors), GMP (granulocyte/monocyte progenitors) and MEP (megakaryocyte/erythrocyte progenitors) are reduced significantly in *Dot1L* KO mice compared with the WT mice<sup>15</sup>. Moreover, an interesting phenomenon in our research was that there was a specific defect in erythroid lineage development in *Dot1L* mutant yolk sac cells, while the myeloid lineage was not significantly affected<sup>7</sup>. However in the *Dot1L* conditional KO adult mice, both myeloid and erythroid lineages were significantly affected<sup>15</sup>. We speculated the possible explanations for the inconsistency. First, a different method was used to generate the *Dot1L* mutant mouse line. The conditional KO described by the other two groups was generated using the Cre-Lox system in which exon 2 on *Dot1l* locus was inducibly deleted by addition of TAM. The gene trap mutation used in our research resulted in an interruption of entire 3' of the gene, after exon 13. Therefore different protein products could be produced through different methods. The difference in protein structure could give rise to distinct biological effect. Second, it is possible that DOT1L plays distinct roles at different developmental stages. Maybe for hematopoiesis during embryonic development, DOT1L is mostly required for erythroid lineage development. The function of DOT1L in myeloid lineage development is marginal. However, for hematopoiesis in the adult stage, DOT1L may play a more extensive role in HSC, multipotent progenitors, and committed erythroid and myeloid progenitors. This different functionality at different stages could be achieved by



regulating the expression of different sets of genes. To test this possibility, genome-wide gene expression analysis in hematopoietic tissues in both embryo and adult should be carried out. By doing this, we will find out whether DOT1L plays different role by controlling expression of different genes at various stages. More attention should be paid on genes that are important for emergence of HSC and the transition from HSC to hematopoietic progenitor cells, and on genes that are important for lineage commitment determination, such as *Scl*, *Runx1*, *Gata2*, *Pu.1*<sup>5,33,134</sup>.

Although anemia is very obvious and severe in *Dot1L* KO mouse embryos, it is not the only defect observed. Our group as well as other groups has also identified diverse impairments including growth retardation, yolk sac angiogenesis defects, and cardiac dilation<sup>7,12</sup>. In order to explore a role of DOT1L in the heart, a cardiac specific conditional KO mouse model has been generated. It was shown that loss-of-function of DOT1L in cardiomyocytes caused heart dilation and postnatal lethality, but DOT1L deficiency in the cardiac organ alone, was not sufficient to cause embryonic lethality<sup>114</sup>. It would be very interesting if hematopoietic-specific conditional KO mice could be established to analyze whether hematopoietic defect alone is responsible for the embryonic lethality.

## Chapter 4

### Mechanistic studies of DOT1L in embryonic erythropoiesis

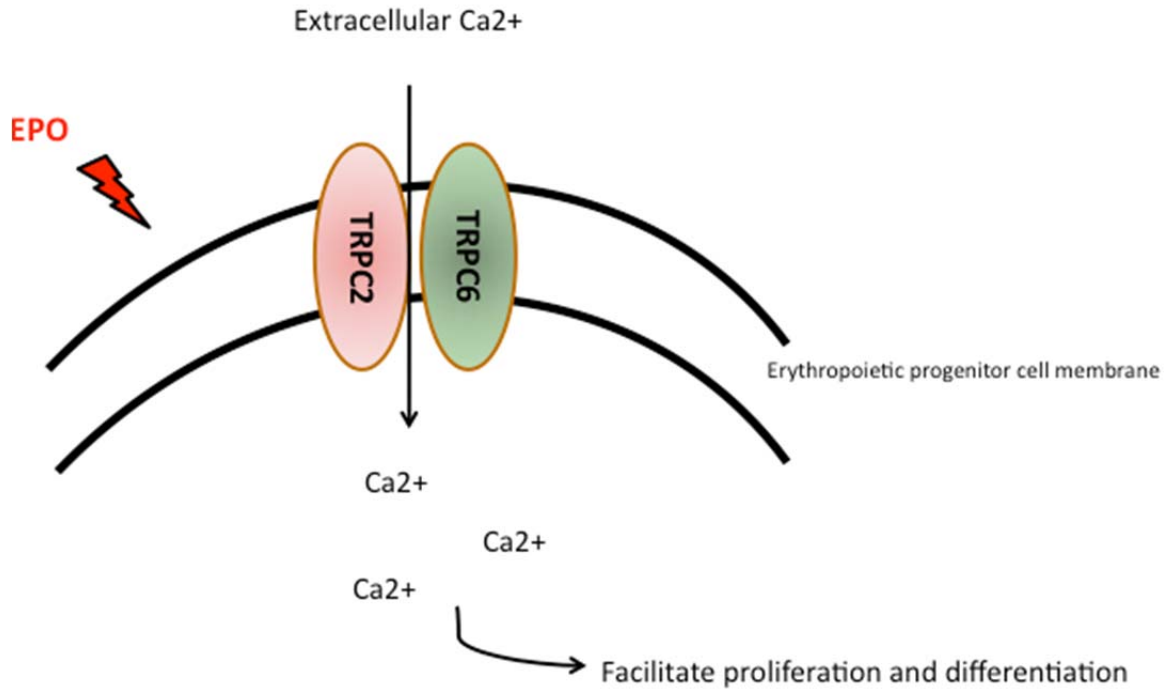
#### 4.1 Introduction

Since our data clearly demonstrated that *Dot1L* deficiency results in an embryonic hematopoietic defect, specifically in the erythroid lineage, I next wanted to find out the possible mechanism(s) through which DOT1L plays its role in hematopoiesis. The differentiation of multi-potential hematopoietic progenitor cells into diverse blood cell types is intricately regulated by specific signaling stimuli and by complex transcriptional networks. Dysfunctional regulation of signaling pathways or of transcriptional programs can result in the development and progression of anemia and/or certain leukemias<sup>4,5</sup>. DOT1L and H3K79 methylation have been proposed to play a role in gene transcriptional regulation<sup>92</sup>. Both DOT1L and H3K79 methylation were found enriched at actively transcribed sites. A strong similarity between the patterning of H3K79 methylation and that of H3K4 methylation, another type of epigenetic modification associated with actively expressed genes, was identified in several mammalian cell lines, suggesting that parallel pathways may exist to specify gene activity or antagonizing gene silencing<sup>13</sup>. Therefore I initiated the mechanistic studies by analyzing the expression pattern changes of some important genes in hematopoiesis upon *Dot1L* mutation.

Previous studies indicated that although H3K79 methylation is a universal modification in the genome, only certain genes requires DOT1L for proper expression<sup>13,92,108</sup>. On the basis of observations across different species, it has been proposed that DOT1L is required by a subset of developmentally regulated genes. We

observed the same phenomenon in our gene expression analyses. Among more than 20 genes that we studied, most of them were not affected by *Dot1L* mutation at a transcriptional level. Interestingly, we observed that the expression of two important transcription factors, *Gata-2* and *pu.1*, were differentially regulated in *Dot1L* KO hematopoietic progenitor cells<sup>7</sup>. The relationship between *Gata-2* and *pu.1* expression has been characterized and established for many years. GATA family factors and PU.1 are lineage-specific transcription factors that are expressed in multi-potential progenitor cells. During lineage commitment, activation of specific transcription factors has been shown to direct precursor cells to a certain differentiation pathway. At the same time, regulators promoting other lineages should be inhibited to ensure normal lineage differentiation<sup>46,52,53</sup>. *Pu.1* is critical for myeloid lineage development and it is essential to block *pu.1* function for normal erythroid cell development<sup>50,53,54</sup>. *Gata-2* and *Gata-1* are important for erythroid lineage development. Both of these genes are repressed when progenitor cells go through a myeloid differentiation pathway, during which *pu.1* is up-regulated<sup>49,53</sup>. Based on previous data and our gene expression analysis, I came up with a first hypothesis about the mechanism through which DOT1L controls embryonic hematopoiesis. Experimental results with regards to this hypothesis will be discussed in the following sections.

Through gene expression analysis, I also found that the expression of *Trpc6*, the gene encoding a member of the transient receptor potential channels (TRPC) family, was dramatically reduced in *Dot1L* mutant progenitor cells, while other TRPC family members were not affected by *Dot1L* deficiency. In erythroid lineage cells, only *Trpc2* and *Trpc6* are expressed<sup>85</sup>. The function of TRPC2 is to facilitate calcium influx in



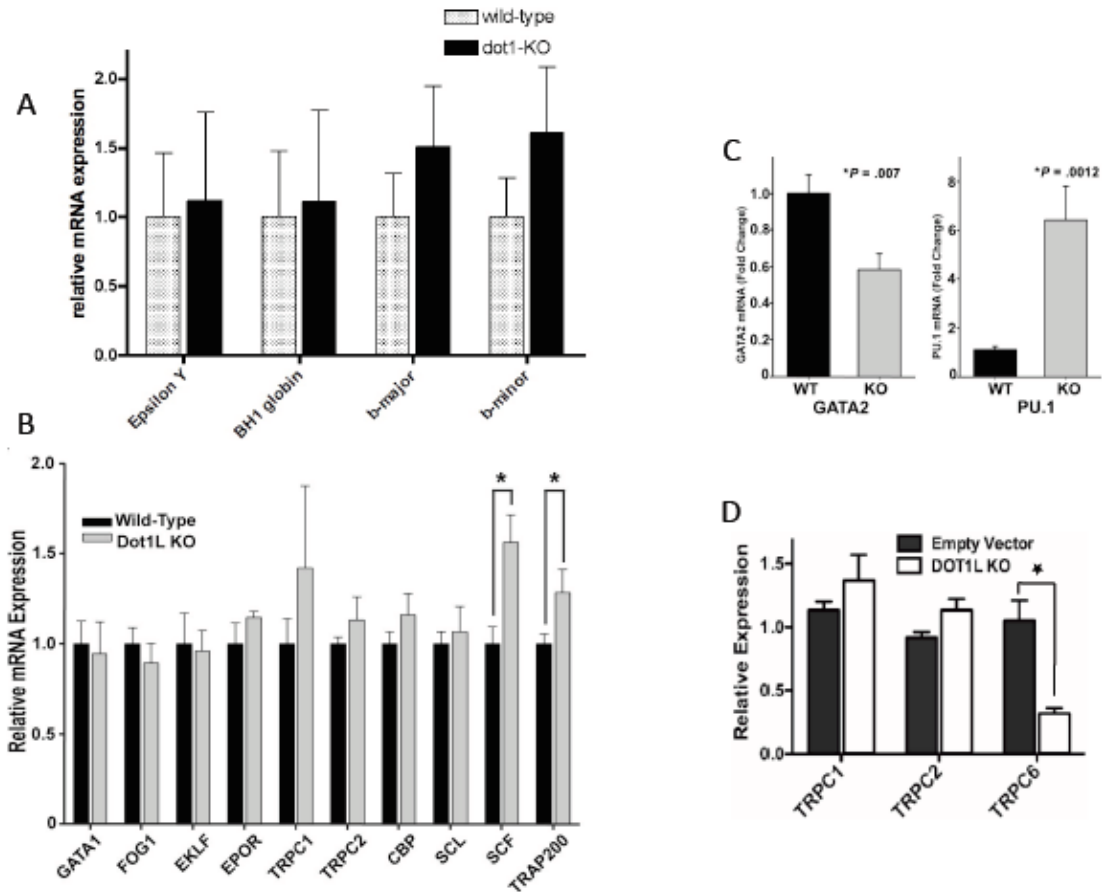
**Figure 4.1 Calcium influx in response to EPO.** In mice, *Trpc2* and *Trpc6* are expressed in erythropoietic lineage. TRPC2 was shown to mediate calcium influx in response to EPO, whereas TRPC6, which interacts with TRPC2, suppresses the EPO-induced calcium influx. Thus, the TRPC2/TRPC6 ratio in the plasma membrane provides a mechanism by which the intracellular calcium can be precisely regulated. The expression change of either of TRPC2 or TRPC6 on the hematopoietic progenitor cells will change the proper ratio of these two factors on cell membrane. If this occurs, the progenitor cells will not be able to respond to EPO properly. The model was drawn based on the results from Chu et al, 2004<sup>85</sup>.

response to erythropoietin, an important signaling mechanism controlling the proliferation and differentiation of erythroid progenitors and precursors. The function of TRPC6 is to inhibit calcium influx through TRPC2. It has been proposed that the stoichiometry of expression of *Trpc2* and *Trpc6* may be an important mechanism for regulating calcium influx in erythroid cells in response to erythropoietin stimulation<sup>74,83-85</sup> (Figure 4.1). In *Dot1L* mutant progenitor cells, the expression of *Trpc6* decreased, while *Trpc2* remained unaffected. We propose that the normal ratio between these two protein factors has been disrupted upon *Dot1L* deficiency. Based on these previous data, a second hypothesis about how DOT1L plays its role in hematopoiesis was proposed and will be discussed in this chapter. These studies explored the possible mechanisms through which DOT1L regulates embryonic hematopoiesis and provide potential explanations for the hematopoietic defect in *Dot1L* deficient animals.

## 4.2 Results

4.2.1 Expression patterns of certain genes that are critical in hematopoiesis are changed in *Dot1L* mutant progenitor cells

To more precisely understand the mechanism by which DOT1L affects erythropoiesis, the expression levels of genes known to play important roles in erythroid development were examined in c-kit positive WT and KO E10.5 yolk sac cells. No significant changes in the expression of globin genes was observed in *Dot1L* mutant cells (Figure 4.2 A), which is consistent with positive benzidine staining of KO erythroid colonies (Figure 3.6). Remarkably, among the genes profiled by reverse transcription-PCR, only five were significantly affected by DOT1L loss: *Gata-2* and *Trpc6*, which were reduced; and *pu.1*, *scf*, and *Trap220*, which were all increased (Figure 4.2 B-D).



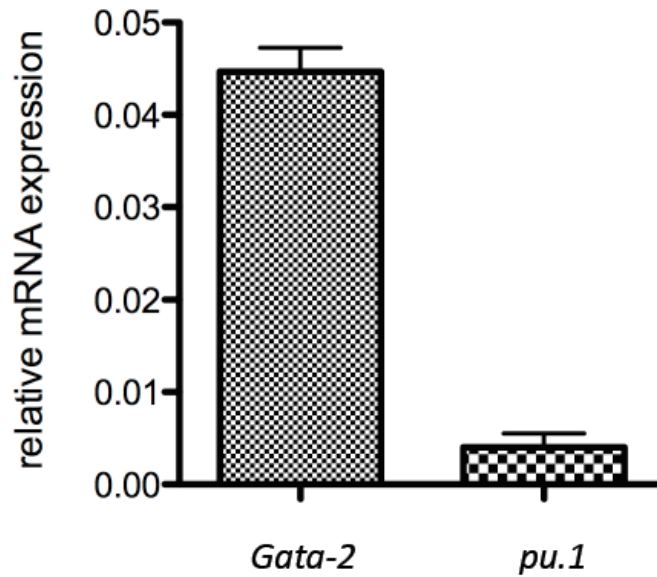
**Figure 4.2. Gene expression analysis of wildtype and *Dot1L* knockout progenitor cells in yolk sac.**

mRNA was extracted from FACS-sorted, c-kit<sup>+</sup> yolk sac progenitor cells. Reverse transcription was performed and genes known to be important in erythropoiesis were assessed by quantitative RT-PCR. mRNA was obtained from c-kit<sup>+</sup>, sorted cells from 3-10 individual yolk sacs. (\*p<0.01; t-test).

The *Dot1L* mutant c-kit<sup>+</sup> hematopoietic progenitors showed a consistent elevation in *pu.1* level relative to WT, whereas *Gata-2* expression was decreased by about 40% (Figure 4.2 C). These data suggest that the maintenance of a normal ratio of *Gata-2* to *pu.1* is influenced by H3K79 methylation and are consistent with the reported reciprocal expression and functional relationship between *Gata-2* and *pu.1* within hematopoietic progenitors<sup>49,53</sup>.

*Trpc6* expression in *Dot1L* mutant progenitor cells was reduced to about 30% of the WT level (Figure 4.2 D). TRPC6 is a calcium permeable non-selective cation channel expressed in brain, smooth muscle containing tissues, as well as hematopoietic cells. It belongs to a transient receptor potential (TRP) protein superfamily. They are voltage-independent calcium channels that can be agonist-regulated<sup>135</sup>. In mouse erythropoietic progenitor cells, erythropoietin can modulate calcium influx through one of these channels, TRPC2<sup>83,84</sup>. The function of TRPC6, however, is to inhibit calcium influx through TRPC2<sup>85</sup>. These data suggest that balance between TRPC2 and TRPC6 is disrupted in *Dot1L* mutant progenitor cells, which may contribute to the erythropoietic defects in CFU assays as well as the anemia observed *in vivo*. It is likely that TRPC6 deficiency results in cells that are defective in response to EPO and die. This death depletes erythroid progenitors and results in the anemia.

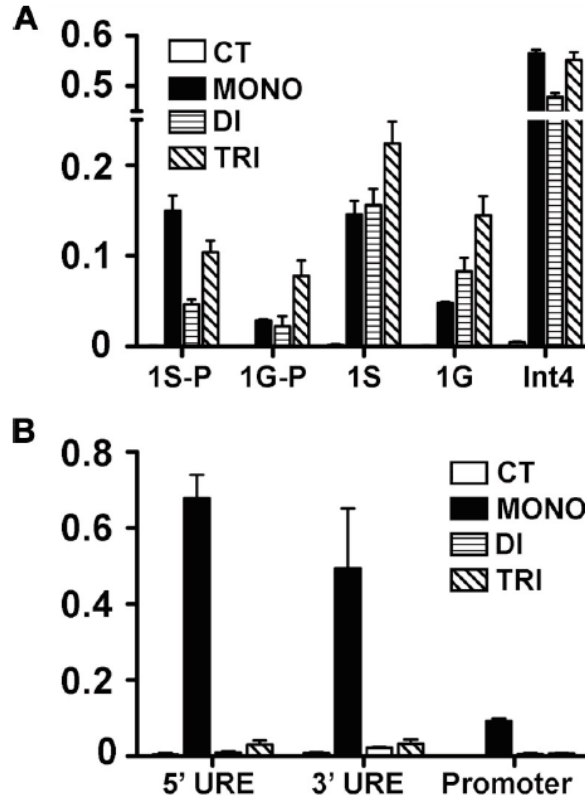
Because *Dot1L* deficiency affected *Gata-2*, *pu.1* and *Trpc6* expression, we sought to determine the H3K79 methylation status within each locus with the use of ChIP analysis. To accumulate chromatin sufficient for this analysis, we used G1E cells<sup>136</sup>, a proerythroblast line that express high levels of *Gata-2* and low levels of *pu.1* (Figure 4.3). We found that in the actively transcribed *Gata-2* locus, H3K79 mono-, di-, and tri-



**Figure 4.3** *Gata-2* and *pu.1* expression in G1E cells

We analyzed *Gata-2* and *pu.1* mRNA levels by RT-PCR in G1E cells. Expression of these genes relative to GAPDH is shown here. *Gata-2* mRNA is abundant in these cells, suggesting its active transcription. In contrast, *pu.1* mRNA is virtually undetectable in these cells.



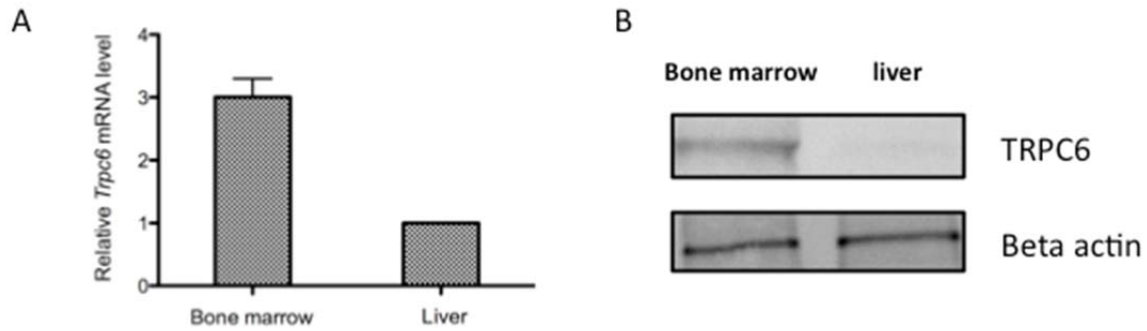


**Figure 4.4. H3K79 methylation in the *Gata-2* and *Pu1/Sfi1* loci.**

ChIP was performed on chromatin from the G1E cell line to assess H3K79 mono-, di-, and trimethylation of promoter and regulatory sites of *Gata-2* and *Pu1/Sfi1* loci in G1E cells. At the *Gata-2* locus (**A**), which is actively transcribed, the 1S and 1G promoters (1S-P, 1G-P) and the first exon following each promoter (1S, 1G), as well as a regulatory element in intron4 (Int4) were examined. At the silenced *Pu1/Sfi1* locus (**B**), we examined 2 upstream regulatory elements, 5'URE and 3'URE, as well as the promoter region. The data represent three independent experiments.

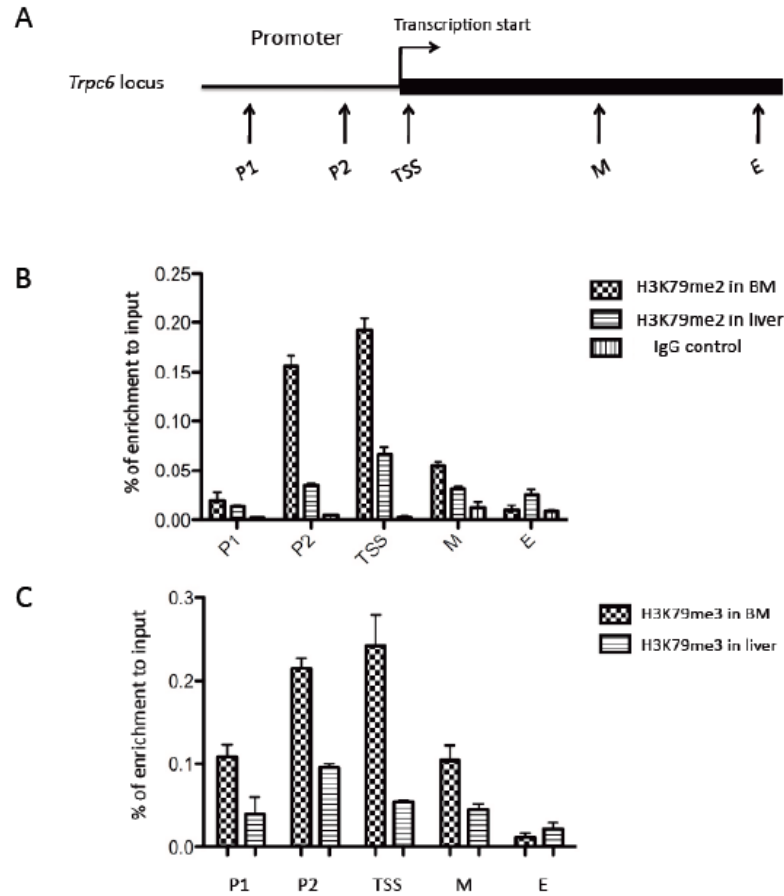
methylation are all enriched at the 1S promoter, the first exon after 1S, the 1G promoter, the first exon after 1G, and intron 4, which contains an important regulatory element (Figure 4.4 A). In the silenced *pu.1* locus, we found that only mono-methylation was enriched in regulatory elements and in the promoter (Figure 4.4 B). We did not find di- and tri-methylation enrichment on these sites.

For the analysis of the *Trpc6* locus, bone marrow and liver were used as examples of high TRPC6 and low TRPC6 expression, respectively<sup>137,138</sup> (Figure 4.5). Several pairs of primers were designed for ChIP, that cover the entire *Trpc6* locus (including the promoter region, the transcription start site, the middle and the end of the gene locus) (Figure 4.6 A). The results of ChIP showed that at the promoter region and transcription start site of *Trpc6*, H3K79 di- and tri-methylation are more enriched in bone marrow cells (where *Trpc6* expression levels are relatively high), than in the liver cells (relatively low expression levels of TRPC6). In the middle of the *Trpc6* locus, H3K79 di- and tri-methylation enrichment is still higher in bone marrow cells, although not significant compared to liver cells. At the end of gene locus, there is nearly no difference in H3K79 methylation between bone marrow and liver cells (Figure 4.6 B, C). These ChIP data on the *Gata-2*, *pu.1* and *Trpc6* loci do not directly implicate H3K79 methylation in regulating transcriptional activity of these genes, however they do correlate with the expression of these genes, and suggests a role for the histone mark in the regulation of their transcription. These results are in accord with previous studies showing H3K79 di- and tri-methylation are associated with regions of active gene expression, whereas mono-methylation is indicative of gene regulatory elements<sup>13</sup>.



**Figure 4.5 *Trpc6* expression in bone marrow and liver.**

(A) *Trpc6* expression was tested by RT-PCR in bone marrow and liver cells. Expression level was shown by  $\Delta\Delta C_t$  method. *Trpc6* mRNA was relatively abundant in bone marrow, suggesting its active transcription. (B) TRPC6 protein was tested by Western Blot in bone marrow and liver cells. It showed that TRPC6 was expressed in bone marrow, but was barely detectable in liver cells.



**Figure 4.6 H3K79 methylation in *Trpc6* locus.**

ChIP was performed on chromatin from bone marrow (BM) and liver cells to assess H3K79 di- and tri-methylation in *Trpc6* locus. (A) Several pairs of primers covering *Trpc6* locus were designed. Their location relative to the locus was shown. H3K79 methylation in promoter region (P1, P2), transcription start site (TSS), middle and end of gene locus (M, E) was analyzed. H3K79 di-methylation (B) and tri-methylation (C) were examined in *Trpc6* locus. The data represent three independent experiments.

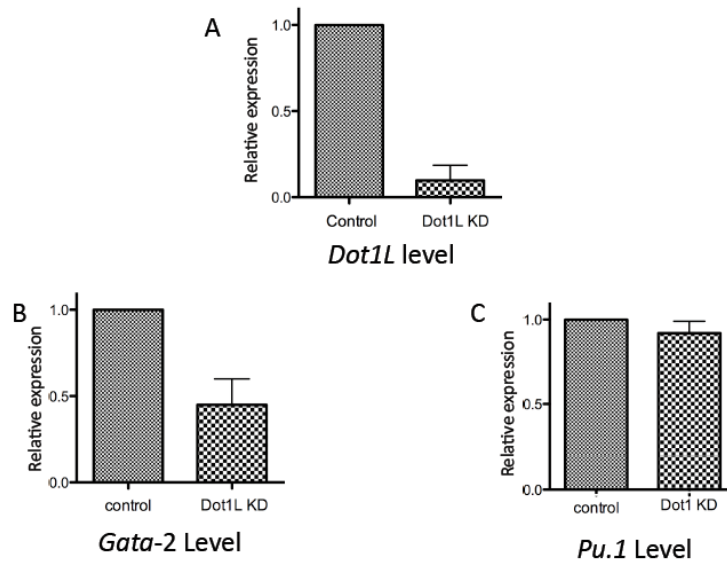
4.2.2 *Gata-2* expression is regulated by DOT1L, but this is not the mechanism by which DOT1L regulates hematopoiesis

In the gene expression study using c-kit<sup>+</sup> yolk sac cells, one interesting finding was that the expression level of *Gata-2* and *pu.1* was differentially regulated in *Dot1L* KO samples. In *Dot1L* KO yolk sac progenitor cells, *Gata-2* expression level reduced to about 50% of WT levels. At the same time, *pu.1* expression increased several fold. Since these transcription factors play critical roles in different lineage development, my first hypothesis was that DOT1L is required for maintaining normal levels of *Gata-2* and *pu.1* in the hematopoietic progenitor cells. When *Dot1L* is mutated, it will directly result in transcription pattern changes in *Gata-2* and *pu.1*, which in turn causes a specific erythroid development defect.

To test the validity of this hypothesis, I first need to establish that expression changes of *Gata-2* and *pu.1* in the *Dot1L* KO progenitor cells is directly controlled by DOT1L expression. Also, I need to show that the expression changes of *Gata-2* and *pu.1* are sufficient to cause erythroid defects to some extent. To this end, different cell lines were used as model systems in this analysis. The first step was trying to knock down *Dot1L* to determine whether *Gata-2* and *pu.1* expression levels were affected. The first cell line I tried was G1E, a genetically-engineered hematopoietic cell line. G1E cells are immortalized GATA-1 null erythroblasts derived from gene-targeted embryonic stem cells. They have been widely used in analyzing transcriptional regulation during erythropoiesis<sup>136</sup>. I tried both transfection of G1E cells with shRNA plasmid (pGIPz) targeted to mDot1L and lentiviral transduction of G1E cells, but neither of them worked. For instance, the percentage of cells expressing the lentiviral reporter, GFP, was

extremely low after two days of transfection with shRNA plasmid, even if I tried different protocols. In the lentiviral transduction experiments, although I obtained a relatively high amount of GFP+ G1E cells, *Dot1L* was not successfully knocked down. We speculate that the possible reason for this unsuccessful knock down in G1E cells is that DOT1L is absolutely required for the survival of this cell line. It is likely that when *Dot1L* level of certain G1E cells in a population is down regulated, critical signaling pathways, such as EPO signaling, are affected. Then the cells with defective DOT1L function will have severe defect in their growth, and will be selected against. However the cells in the same population with relatively normal *Dot1L* function will soon become dominant. Therefore, it is not suitable to use G1E cells as a model system for study of DOT1L function.

Another cell line, K562 cell was also used to test *Dot1L* knock down. Developmentally, K562 cells are more primitive in nature than G1E cells and behave more like undifferentiated early hematopoietic multi-potent progenitor cells<sup>4</sup>. It turned out it was much easier to do the transfection and to knock down *Dot1L* expression in K562 cells than in G1E cells. Using the pGIPz shRNA plasmid against human *Dot1L*, *Dot1L* transcription levels were knocked down to about 20% of the control group (Figure 4.7 A). In order to determine whether *Gata-2* and *pu.1* expression is regulated DOT1L, real-time PCR was performed to test *Gata-2* and *pu.1* levels in *Dot1L* KO K562 cells. A 50% reduction of *Gata-2* levels was observed, a result which was quite similar in magnitude to our *ex vivo* study using yolk sac progenitor cells. However, *pu.1* levels remained unchanged in all the experiments using *Dot1L* deficient K562 cells (Figure 4.7 B, C). In order to verify this result, I repeated the experiment by transfection of K562



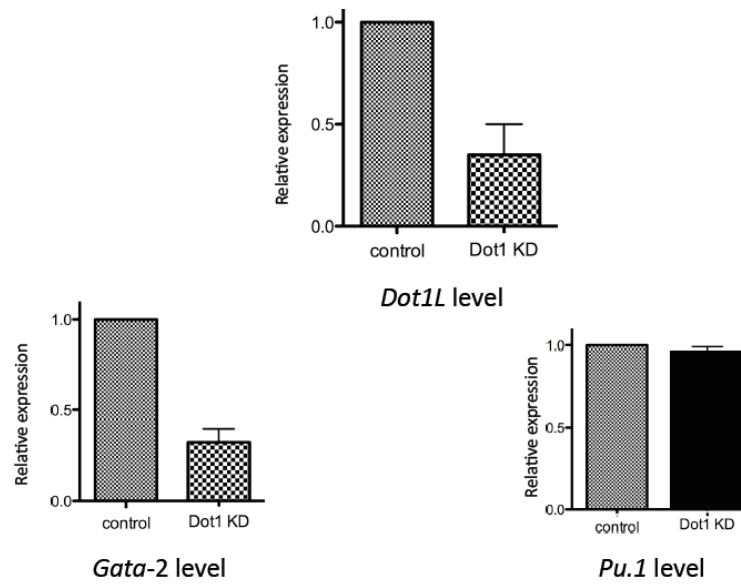
**Figure 4.7 *Dot1* knock down with pGIPz vector changes the expression level of *Gata-2*.**

(A). K562 cells were transfected with pGIPz vector containing sequence for shRNA against *hDot1L* or scrambled sequence as control. *Dot1L* level was knocked down to less than 20% compared to control. (B). when *Dot1L* was knocked down. *Gata-2* level was reduced to about 40%. C. *Dot1L* KD did not change *pu.1* level in K562 cells.

cells with pLKO.1 shRNA plasmid. As expected, *Dot1L* level was down regulated to about 30% of the control group. A 50-60% reduction of *Gata-2* expression was observed again. However, the *pu.1* level was still not affected in *Dot1L* knock down in these K562 cells (Figure 4.8). These experiments indicate that only the transcription of *Gata-2* is regulated by DOT1L directly. However the *pu.1* transcription is not directly influenced by DOT1L level. Although previous study showed that *Gata-2* deficiency induces *pu.1* expression<sup>49</sup>, we did not observe that in our study. Thus, the result in the K562 cells is only partially in accord with gene expression study in yolk sac progenitor cells, and my hypothesis is not completely supported by the results.

Since only *Gata-2* expression seems to be reduced in *Dot1L* deficient cells, it is worthwhile to test whether *Gata-2* reduction alone is able to cause hematopoietic defects. *Gata-2* is broadly expressed in the hematopoietic system. It is expressed at particularly high levels in hematopoietic progenitor cells as well as in megakaryocyte lineages<sup>45</sup>. The function of GATA-2 has been studied using different model systems. A *Gata-2* KO mouse showed panhematopoietic defects and embryonic lethality by E12.5<sup>45</sup>. ES cells with deficient *Gata-2* function has been used in the *in vivo* and *in vitro* differentiation studies and it is required for proliferation and survival of immature hematopoietic progenitors<sup>47</sup>. In our research, *Gata-2* levels in *Dot1L* KO progenitor cells was reduced to about 50% of the wild type. This is quite similar to the expression level of *Gata-2* heterozygous hematopoietic progenitor cells. I wanted to use *Gata-2*<sup>+/-</sup> progenitor cells as model system to investigate how reduction of GATA-2 influences hematopoiesis. The effect of haploinsufficiency of *Gata-2* on hematopoietic development is not very well defined. In an early study published in *Nature* in 1994, the hematopoietic potential of

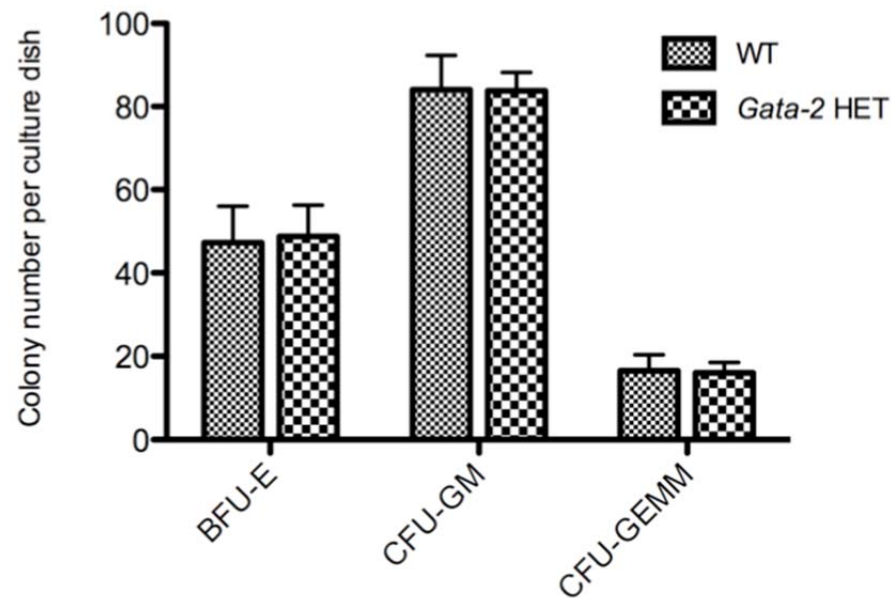




**Figure 4.8 *Dot1* knock down with pLKO.1 vector.**

(A). K562 cells were transfected with pLKO.1 vector containing sequence for shRNA against *hDot1L* or scrambled sequence as control. *Dot1L* expression level was knocked down 70% compared to control. (B). when *Dot1L* was knocked down, *Gata-2* level was reduced about 60%. C. *Dot1L* KD did not change *pu.1* level in K562 cells.

*Gata-2* +/- yolk sac progenitor cells was analyzed. No difference in hematopoietic potential between *Gata-2* ++ and *Gata-2* +/- was observed. They had equal abilities to give rise to erythroid and myeloid colonies<sup>45</sup>. In contrast, another study analyzing GATA-2 function in adult hematopoiesis showed opposite results. First, *Gata-2* +/- progenitor cells demonstrated decreased blood production in colony-forming assays; second, the stem cell compartment in the bone marrow of *Gata-2* +/- mice showed more significant changes, including a lower abundance of Lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>-</sup> cells and a poor performance in competitive transplantation assays; third, a stem cell-enriched population from *Gata-2* +/- bone marrow exhibited a greater frequency of apoptotic cells associated with decreased expression of the anti-apoptotic gene *Bcl-xL*<sup>139</sup>. Since these previous experiments regarding the hematopoietic potential of *Gata-2* +/- progenitor cells are apparently contradictory, I set up colony forming unit assays to directly test the hypothesis. My experiments showed that the *Gata-2* +/- yolk sac progenitor cells can give rise to both erythroid colonies (BFU-E), myeloid colonies (CFU-GM), and mixed colonies (CFU-GEMM) as efficiently as wild type control progenitors (Figure 4.9). No changes in the morphology of the *Gata-2* +/- colonies were observed. Our experiments were in accord with one of the previous studies<sup>45</sup>, and suggested that at least at the embryonic stage, a 50% reduction of *Gata-2* will not generate an observable hematopoietic defect. All together, our results indicate that while *Gata-2* expression is controlled by DOT1L levels, *pu.1* expression is not influenced by DOT1L. Thus, the reduction of *Gata-2* alone may not be the major reason for the hematopoietic defect in *Dot1L* deficient embryos.



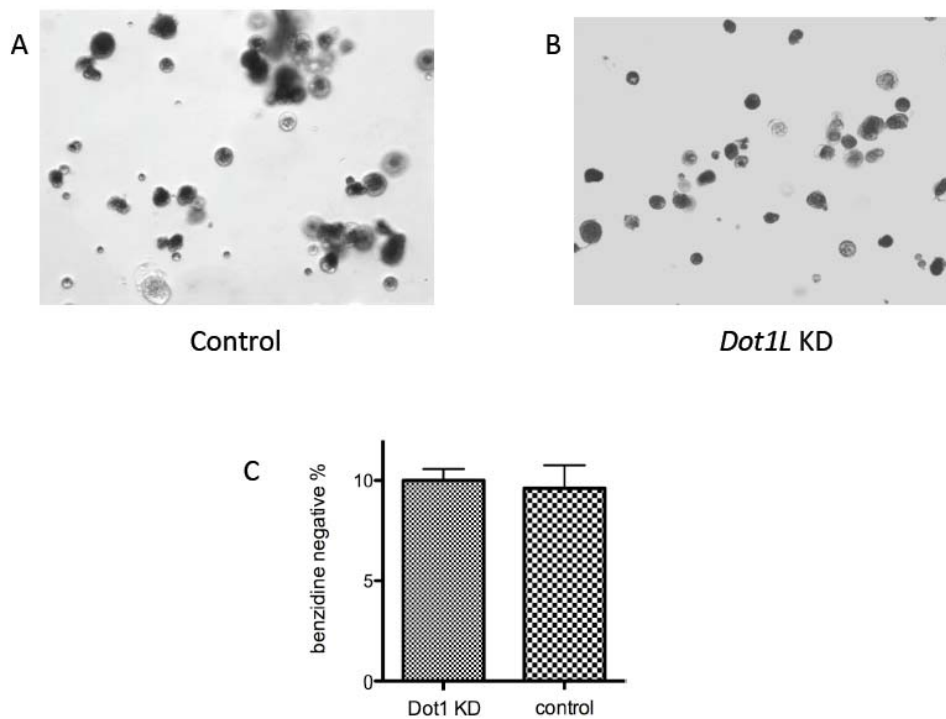
**Figure 4.9 *Gata-2* +/- yolk sac progenitors did not show hematopoietic defect in CFU assay.**

Identical number of dissociated yolk sac cells from wild type and *Gata-2* +/- E10.5 embryos were cultured in methylcellulose growth medium supporting both erythroid and granulocyte-macrophage progenitors. *Gata-2* +/- progenitors had the same potential as wild type to give rise to both erythroid and myeloid lineage colonies.

The developmental program of erythropoiesis includes at least two components: the commitment of progenitor cells and hemoglobinization. K562 cells have been used as a model for hemoglobin switching in previous studies<sup>140</sup>. One important feature of K562 cells is that upon treatment with hemin or other inducing agents, K562 cells are induced to differentiate into hemoglobin-producing cells<sup>140,141</sup>. In our gene expression analysis of yolk sac progenitor cells, it was shown that *Dot1L* KO does not change the expression pattern of hemoglobin genes at the transcription level. Since I had *Dot1L* deficient K562 cells available, I wanted to find out whether the lack of DOT1L affects the process of hemoglobin production. When *Dot1L* deficient K562 cells and control cells were treated with hemin for 36-48 hours, both groups produced about 90% benzidine-positive hemoglobin containing cells (Figure 4.10), a result consistent with our previous benzidine staining test in the CFU assay (Figure 3.6). This result indicated that *Dot1L* deficiency does not affect hemoglobin generation at either the transcriptional or translational stages. The anemia *in vivo* and hematopoietic defects *in vitro* in the *Dot1L* KO samples, therefore, could not be attributed to defective hemoglobin production.

4.2.3 *Trpc6* expression is affected in *Dot1L* deficient yolk sac progenitor cells and this may contribute to the erythropoietic defect

As discussed before, the first hypothesis about *Gata-2/pu.1* transcription regulation by DOT1L was not supported by the studies. In this section, the second proposed mechanism is addressed. Since the defect is largely limited to erythroid lineage development, I reasoned that the failure to form large erythroid colonies *in vitro* and anemia *in vivo* could be due to inappropriate responses to erythroid growth stimuli. Many factors such as cytokines and nuclear hormones contribute to the process of



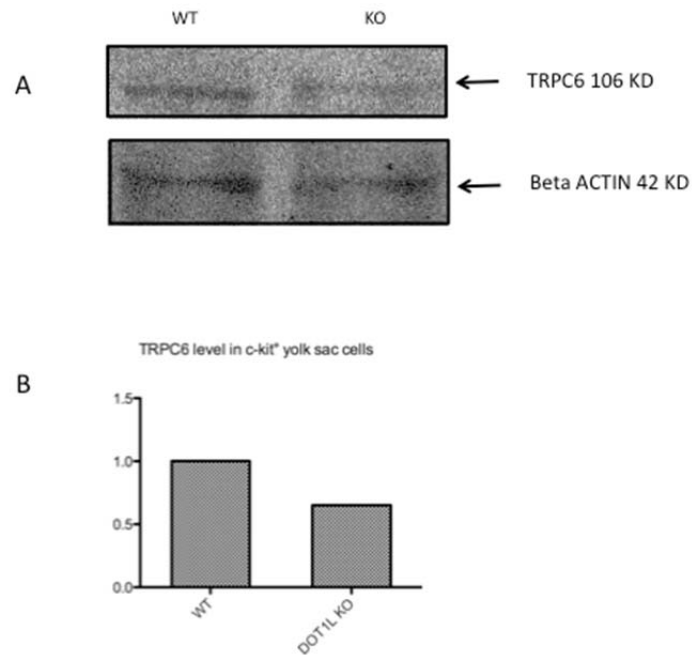
**Figure 4.10 Hemin induction on K562 cells.**

(A-B). K562 cells transfected with pLKO.1 vector harboring scrambled sequence (A) or shRNA sequence against *hDot1L* (B) were cultured in medium containing 50  $\mu$ M Hemin for 48 hours. Cells were stained with Benzidine to show hemoglobin expression. (C). Cells were counted on hemocytometer. It is shown that the percentage of hemoglobin-containing cells in control and KD group were almost the same.

erythropoiesis<sup>4</sup>. For example, both interleukin-3 and Stem Cell Factor can raise the number of cells within BFU-E and CFU-E colonies. Insulin and Insulin-Like Growth Factor-I play a role at the latter stages of erythroid maturation<sup>6</sup>. Among all these factors, erythropoietin (EPO) plays a central role in erythropoiesis through the promotion of proliferation, differentiation, and survival of erythroid precursors, including CFU-E and BFU-E<sup>6</sup>. When EPO binds to its specific receptor on the cell surface, several signaling pathways are initiated<sup>71</sup>. One important function of EPO is to increase intracellular calcium in primary erythroid cells, which is an important signaling mechanism controlling the proliferation and differentiation of progenitors and precursors<sup>75,131</sup>. It has been shown that the calcium influx in response to EPO is mediated by transient receptor potential channels (TRPC)<sup>83</sup>. In erythroid cells, only two of the family members, *Trpc2* and *Trpc6*, are expressed and the stoichiometry of TRPC2 and TRPC6 proteins can be an important mechanism for regulating calcium influx in erythroid cells in response to EPO stimulation<sup>84,85</sup>. Interestingly, in the gene expression analysis in yolk sac progenitor cells, I found that at transcription level, only *Trpc6* expression decreased significantly in *Dot1L* KO samples, while other TRPC members, including *Trpc2*, were not affected by *Dot1L* mutation. Therefore the balance between *Trpc2* and *Trpc6* is disrupted in *Dot1L* KO progenitor cells. Based on the results from other research groups and my own findings, my second hypothesis is that *Dot1L* deficiency results in down regulation of *Trpc6*. The change of stoichiometry of TRPC6 and TRPC2 in turn, results in abnormal calcium influx in response to EPO during erythropoiesis, which leads to a relatively specific erythropoietic defect.

In the previous section, *Trpc6* expression at the transcriptional level was shown to be decreased significantly in *Dot1L* KO yolk sac progenitor cells. It is necessary to find out whether *Trpc6* expression at the translational level was also affected by *Dot1L* mutation. Since the protein amount from the progenitor cells of a single yolk sac was very little, c-kit<sup>+</sup> yolk sac cells of the same genotypes were pooled together to perform Western Blot (Figure 4.11). Due to low protein concentration, the signal on the Western Blots was not very strong. By doing quantification using ImageJ software, we determined that TRPC6 levels in *Dot1L* KO progenitors was reduced by about 40% compared to the WT group. This result was consistent with previous RT-PCR results and supported my hypothesis, indicating that *Dot1L* deficiency indeed changes *Trpc6* expression.

Next, it was important to find out whether calcium influx in response to EPO was changed in *Dot1L* KO hematopoietic progenitors due to the reduction of *Trpc6* levels. Calcium is a ubiquitous intracellular signal responsible for controlling numerous cellular processes. The extracellular concentrations of calcium in most cells are generally quite high, 3-4mM. However, the concentrations inside the cell are tightly controlled and usually kept at or below 100nM. Different types of stimuli will generate the calcium mobilizing signals, which increase intracellular calcium level to around 1000nM. The increased calcium level will create a wide range of signals that play critical roles in biological processes as diverse as fertilization, proliferation, development, contraction and secretion<sup>79</sup>. Importantly, though calcium signaling is important and necessary, it is also potentially toxic to the cells. Intracellular free calcium is a key element responsible for a number of calcium-dependent apoptosis pathways<sup>78</sup>. Therefore, a balance of calcium and homeostasis is important for both survival as well as normal responsiveness

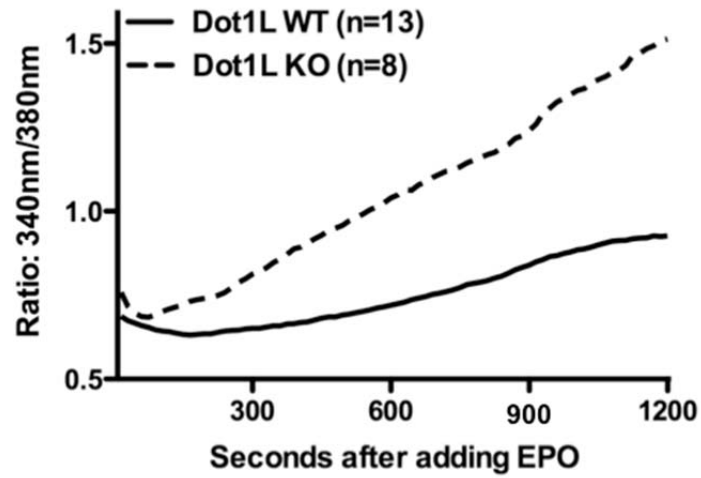


**Figure 4.11 *Dot1L* deficiency results in TRPC6 protein level reduction in c-kit+ yolk sac cells.**

(A) c-kit+ yolk sac cells from 6 individual *Dot1L* KO embryos and 6 individual wild type embryos were pooled together and protein was extracted for Western blot. (B) Western blot image was analyzed by ImageJ software. Signal intensity of *Dot1L* KO group decreased 40% compared with wild type group.



to extracellular signals. Intracellular calcium is measured with the use of different kind of calcium ion probes, such as Fura-2 or Calcium Green-1. Fura-2 was used in my experiments. It is a ratiometric indicator that exhibits a shift in its absorption or excitation peak from 380nm to 340nm upon binding calcium<sup>84</sup>. Whole yolk sacs were dissociated into single cells and were fixed to the bottom of the culture dishes. The yolk sac cells were then stained with Fura-2. At the beginning of the experiment, cells were treated with EPO at 10 U/ml. The calcium signal in individual cell was recorded on a fluorescent microscope for 20 minutes (Figure 4.12). In each yolk sac, the signals of about 30 individual cells were recorded and analyzed. Among these cells, I usually could observe 3 to 4 cells in which the calcium signal changed. The rest of the cells in the group had no response to EPO addition and were not taken into account. This makes sense, because hematopoietic progenitor cells (C-kit+ enriched) constitute only about 10% of the whole yolk sac cells, (as shown in our flow cytometry analysis, Figure 3.8) and they make up the population that is supposed to react to EPO treatment. From figure 4.12, it shows that the response of wild type cells to EPO was relatively slow. After about 5 minutes, the calcium signal started to go up gradually. After about 17 minutes, a plateau was formed on the curve, meaning the intracellular calcium concentration stopped going up. In sharp contrast, the *Dot1L* KO cells responded promptly and the calcium signal went up right after EPO treatment. The slope of the curve was much steeper than that of the wild type, indicating a much faster calcium influx in *Dot1L* KO cells. Even after 17 minutes, the curve did not level off. Thus the final calcium signal in *Dot1L* KO cells after 20 minutes of EPO treatment was much higher than WT cells. This figure is a representative of three experiments using three individual litters. The curve in the figure is not the signal of one



**Figure 4.12 Intracellular calcium change in yolk sac cells upon EPO treatment.**

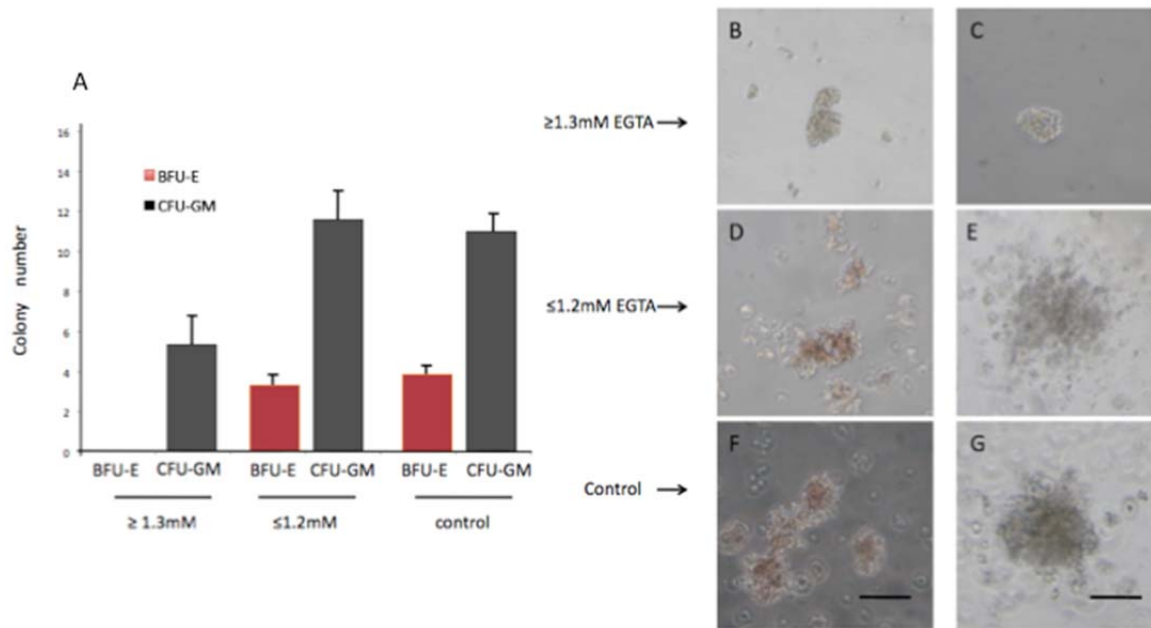
E10.5 *Dot1L* KO and WT yolk sac cells were stained with calcium imaging dye Fura-2 and treated with EPO (10U/ml). Calcium signal in individual cell was recorded by fluorescent microscope for 20 minutes. The curve represented the composite result of multiple cells (KO n=5; WT n=6).

cell, but is the composite curve of several cells from three or more individual yolk sacs of the same genotype. The trend of the curves looked similar in all experiments. In summary, the results from the calcium imaging assays were consistent with previous studies about the function of TRPC2 and TRPC6<sup>85</sup>. Since high intracellular calcium is able to trigger calcium-mediated apoptosis, this result was in accord with our apoptotic analysis with yolk sac cells undergoing erythropoiesis. Also the result supported my second hypothesis, indicating that *Dot1L* deficiency results in abnormal calcium influx in response to EPO; this abnormal response may contribute to the observed erythropoietic defect.

Next, it would be important to test whether the abnormal calcium influx is really the factor for erythropoietic defect in *Dot1L* KO progenitor cells. I reason that if the high calcium concentration does play a critical role in *Dot1L* KO progenitor cells, the hematopoietic defects would be rescued if the abnormally high calcium level is decreased by some means. One of the possible ways is to use the calcium specific chelator to reduce intracellular calcium<sup>142</sup>. Different concentration of calcium chelator in the growth medium would be able to reduce intracellular calcium levels of *Dot1L* KO progenitor cells to different extents. If a wide range of concentrations for the chelator in the medium is attempted, it is possible that certain concentration could be identified that could reduce intracellular calcium of *Dot1L* KO progenitor cells to a level comparable to WT cells, in response to EPO treatment. The erythropoietic defect should be rescued at least to some extent if the calcium concentration is really critical.

Our experiment and studies from others showed that during the first minutes of EPO stimulation, the intracellular calcium level does not increase extensively. These

observations suggested that the calcium level increase is not due to intracellular calcium release. Using HEK 293T cells as model system, it has been shown that extracellular free calcium is the source of calcium increase in response to EPO<sup>74</sup>. EGTA (ethylene glycol tetraacetic acid) has been widely used in many calcium influx analyses<sup>74,143</sup>. EGTA is a chelating agent with higher affinity for calcium than magnesium and it usually exerts its function outside the cells. The divalent calcium ion can be bound by the multiple anionic carboxyl groups of EGTA and the intracellular calcium level can be finally suppressed<sup>144</sup>. Based on previous research, 10mM EGTA is high enough to inhibit calcium influx<sup>143</sup>. The strategy of this experiment was quite straightforward. It was just like a previous CFU assay, except that different amounts of EGTA were added to M3434 medium. I started with 12mM EGTA in the medium, which is supposed to inhibit calcium influx completely, and tested various concentrations all the way down to 1 mM. In each experiment, M3434 without EGTA was used in the control plate. The experiments have been attempted in 8 litters including 13 *DotIL* KO embryos. Although extensive EGTA concentrations were tested, no rescue of the erythropoietic defect in *DotIL* KO progenitor cells was observed at any EGTA concentration. When EGTA is higher than 1.3mM, the colony growth was severely inhibited. There were no erythroid colonies at all. A few small colonies that appeared to be small CFU-GM could be observed on the culture dish. But their colony size was much smaller than CFU-GM in the control group. All concentrations higher than 1.3mM seem to have the similar inhibitory effect. When EGTA was lower than 1.2 mM, it lost its inhibitory effect completely. The colony numbers per dish and colony size were comparable to the control group (Figure 4.13). It appears that EGTA's effect on calcium influx in yolk sac progenitor cells is quite abrupt:



**Figure 4.13 Erythropoietic defect of *Dot1L* progenitor cells can not be rescued by adding EGTA to growth medium.** (A). *Dot1L* KO yolk sac cells were grown in medium containing various concentration of EGTA. When EGTA concentration was higher than 1.3mM (from 1.3mM to 20mM), erythroid colonies were completely inhibited and no recovery was observed. Reduced number of myeloid-like colonies were found. When EGTA concentration was lower than 1.2mM, it lost its potency completely, yielding comparable erythroid colonies and myeloid colonies with control group. . (B-C). Representative pictures of colonies derived from culture with 1.3mM (B) or higher concentration (C) of EGTA. (D-E). Representative pictures of colonies derived from culture with EGTA lower than 1.2mM. Erythroid colony (D) and myeloid colony (E) are shown here. (F-G). Typical erythroid colony (F) and myeloid colony (G) derived from *Dot1L* KO yolk sac cells in M3434 medium without EGTA. (Note: scale bar represents 100um)

either completely inhibitory or completely incompetent. Therefore, EGTA was not a suitable reagent for the rescue experiment of *Dot1L* KO yolk sac progenitor cells.

### 4.3 Discussion

Transcriptional regulation plays a critical role in hematopoietic progenitor cell differentiation and proliferation. Dysregulation of transcriptional mechanisms usually results in anemia and specific leukemias<sup>5</sup>. Since DOT1L has been proposed to play a potential role in gene expression regulation<sup>92</sup>, I initiated the mechanistic studies of DOT1L in hematopoiesis by comparing the expression level of certain critical genes in *Dot1L* KO progenitor cells with wild type progenitor cells. Among more than twenty genes analyzed, only the expression levels of five genes were significantly affected by *Dot1L* mutation: *Gata-2* and *Trpc6*, which were reduced; and *pu.1*, *scf*, and *Trap220*, which were all increased.

Although H3K79 methylation is observed universally throughout the genome, *Dot1L* mutation only results in transcription change in a subset of genes. This interesting phenomenon has been reported by several research groups using different model systems<sup>13,108</sup>. All of the observations led to a hypothesis that only a subset of developmentally regulated genes requires DOT1L for their normal expression. Our results were consistent with these previous studies, indicating that during embryonic hematopoiesis, only certain important genes are affected by *Dot1L* mutation. We predict that the expression pattern changes of these important genes would contribute to the erythropoietic defect.

In my studies, the research has focused on three genes: *Gata-2*, *pu.1*, and *Trpc6*. The reason for this preference is that the expression pattern change of these three genes is

closely associated with the erythropoietic defect phenotype. GATA family factor and PU.1 are lineage-specific transcription factors and play critical role in lineage commitment decisions<sup>53</sup>. *Gata-2* is expressed in hematopoietic stem and progenitor cells and is important for erythroid lineage development. In contrast, *pu.1* is critical for myeloid lineage development and it is essential to suppress *pu.1* function for normal erythroid cell development<sup>50,53,54</sup>. *Trpc6* belongs to a protein family of voltage-independent calcium channel and is expressed specifically in erythroid progenitor cells. It is supposed to play an essential role in calcium regulation in response to EPO during erythropoiesis. Dysregulation of calcium in the progenitor cells will lead to either apoptosis or failure to grow<sup>74,85</sup>.

Although the function of SCF and TRAP220 in hematopoiesis has been well recognized, the expression pattern change of these genes is not likely to result in defects observed in *Dot1L* KO hematopoietic progenitor cells. SCF is critical for erythroid lineage development, since mutation in *scf* or its receptor c-Kit leads to significant reduction of fetal liver CFU-E progenitors and severe anemia<sup>4</sup>. But in our experiments, *scf* expression levels went up in *Dot1L* KO progenitor cells, not quite consistent with the anemia phenotype. TRAP220 deficient progenitor cells also have defect specific in the erythroid, but not the myeloid lineage. More importantly, TRAP220 is also very important in globin gene expression.  $\beta$ -globin expression is abrogated in *Trap220* mutant embryos<sup>5</sup>. In our experiments, however, an increase in *Trap220* expression was observed in *Dot1L* KO progenitor cells, and globin gene expression pattern was not affected. All of the results implied that *Trap220* expression change in *Dot1L* KO progenitor cells may not be responsible for the erythroid defect.

GATA-2 and PU.1 are transcription factors that play critical roles in lineage specific development. My first hypothesis was that DOT1L is required for maintaining the normal level of *Gata-2* and *pu.1* in the hematopoietic progenitor cells. When *Dot1L* is mutated, it will directly result in transcription pattern change of *Gata-2* and *pu.1*, which in turn causes specific erythroid development defect. This hypothesis was not supported by the experimental results. I observed *Gata-2* and *pu.1* differential regulation in *Dot1L* KO progenitor cells and wanted to test whether these two genes were directly regulated by DOT1L in an *in vitro* model. In K562 cells, only *Gata-2* transcription was regulated by DOT1L. *Pu.1* levels were not affected. This is not consistent with our observation in yolk sac progenitor cells. A possible explanation for the gene expression change and erythropoietic defect in *Dot1L* KO progenitors is that *Gata-2* is the only transcriptional regulation target of DOT1L. The higher level of *pu.1* in *Dot1L* KO yolk sac cells is not due to direct transcriptional regulation, but rather is an accumulated result of developmental defect during hematopoiesis. For instance, an erythroid-lineage specific defect will result in predominance of myeloid progenitors in the whole progenitor population, which could change the overall gene expression pattern of yolk sac progenitors.

Since only *Gata-2* expression seems to be regulated by DOT1L, I analyzed whether *Gata-2* reduction alone (haploinsufficiency) is able to cause hematopoietic defects. The effect of *Gata-2* haploinsufficiency on hematopoiesis has been tested in yolk sacs at both the embryonic stage and bone marrow in adult stage<sup>45,139</sup>. My result was consistent with the study carried out in yolk sac cells, showing that a 50% reduction of *Gata-2* alone would not cause observable hematopoietic defects at the embryonic stage.



This result was quite distinct from the study in bone marrow at the adult stage. It is possible that the function of GATA-2 is different at various developmental stages. At the embryonic stage, 50% expression of *Gata-2* is sufficient to maintain normal hematopoietic development. But at the adult stage, GATA-2 becomes more important and the expression of *Gata-2* from both alleles is required for maintaining adult hematopoietic stem cells.

Another important finding in my gene expression studies in yolk sac cells was that *Trpc6* expression was reduced dramatically in *Dot1L* KO progenitors. My second hypothesis was that *Dot1L* deficiency resulted in down regulation of *Trpc6*. The change of stoichiometry of TRPC6 and TRPC2 in turn results in abnormal calcium influx in response to EPO during erythropoiesis, which leads to a specific erythropoietic defect. This hypothesis was supported by some of my experiments. First, *Trpc6* expression is closely associated with DOT1L level. At both the transcriptional and translational level, *Dot1L* KO results in *Trpc6* reduction in the c-kit<sup>+</sup> yolk sac progenitor cells, suggesting DOT1L and H3K79 methylation play a role in *Trpc6* expression regulation. Second, calcium influx in response to EPO was abnormal in *Dot1L* KO yolk sac cells, and this may potentially contribute to the erythroid lineage defect. However, when I tried to rescue the erythroid defect by changing intracellular calcium level in *Dot1L* KO yolk sac cells, no rescue was observed by using EGTA, the calcium chelating agent. The effect of EGTA was either a complete inhibition of erythroid colony formation or no function at all in *Dot1L* KO yolk sac cells.

In a previous study, 5mM EGTA was used to inhibit erythroid colony formation, which was higher than I found<sup>80</sup>. The difference could be due to different methods and

medium used in colony forming assay. In that study, which was performed 30 years ago, erythroid colonies were grown employing the plasma clot technique<sup>80</sup>. In my experiment, MethoCult<sup>®</sup> GF M3434 from StemCell Technology was used. It is optimized for the detection and quantification of mouse hematopoietic progenitors in various tissues. The inhibition of erythroid colony formation was largely due to change of intracellular calcium concentration by EGTA. EGTA is a chelating agent with a much higher affinity for calcium than for magnesium ions. The specificity was shown by the fact that inhibition of erythroid colony formation by EGTA could be rescued by calcium, but not by magnesium, zinc or other ions<sup>80</sup>.

An interesting phenomenon in EGTA chelating experiment was that when erythroid colonies were completely inhibited by EGTA higher than 1.3mM, myeloid colonies could still be observed, although the colonies number and size of these myeloid colonies were significantly reduced compared to the control group. This indicates that erythroid progenitor cells are more sensitive to intracellular calcium concentration changes than myeloid progenitors. This result was consistent with previous data that the regulation of intracellular calcium by EPO was especially critical for the proliferation and differentiation of erythroid progenitors<sup>75,131</sup>. Another thing that should be pointed out is that although small myeloid colonies could be observed in medium containing higher concentrations of EGTA, they were only identified by their morphology. Their identity could be further confirmed by myeloid specific antibody staining, such as Mac-1 and Gr-1.

As I discussed previously, dissociated whole yolk cells were used in the calcium imaging experiment. The yolk sac is a bilayer structure, consisting of mesoderm-derived

and endoderm-derived cell layers<sup>1,18</sup>. In my experiments, the intracellular calcium of only 3-4 cells was changed upon EPO treatment among 30 cells recorded. This is consistent with our previous result that hematopoietic progenitor cells (represented by c-kit+ cells) only account for 6-10% of the whole population. The quality of the experiment would have been improved if enriched hematopoietic progenitor cells in the yolk sac were used.

The erythropoietic defects could not be rescued using EGTA under current experimental conditions. However, the second hypothesis is still supported by some experimental results and looks promising. Other agent should be applied in future studies, such as BAPTA-AM, which exerts its function in a different way from EGTA<sup>145</sup>. BAPTA-AM has been extensively used as an intracellular calcium-specific chelator. It does not bind calcium until it penetrates the cells and is hydrolyzed by cytoplasmic esterases into its active form. Therefore, it will selectively buffer intracellular calcium levels without affecting extracellular concentrations<sup>145</sup>.

## Chapter 5

### General Discussions

A critical role for the DOT1 Histone H3, Lysine 79 (H3K79) methyltransferase in telomeric silencing was first described in *Saccharomyces cerevisiae*<sup>97</sup>. In the subsequent ten years, a vast array of functions have been attributed to the protein, including roles in transcriptional regulation, DNA repair, cell cycle regulation, and embryonic development, in a variety of different model systems, including the fruit fly, various mammalian cell lines, and in mice<sup>10-13,108</sup>. In this dissertation, I focus on the role of the methyltransferase in mouse embryonic hematopoiesis. Precise regulation of H3K79 methylation is absolutely required for mouse embryonic development and deletion of *Dot1L* results in embryonic lethality at around mid-gestation<sup>7</sup>. In these initial studies, defective vascular development was reported, but no clear reason for the lethality was described<sup>12</sup>. In order to perform a more in depth mechanistic analysis of *Dot1L*'s function during this period of embryonic development, with the goal of obtaining greater insight into the potential cause of the lethality, a gene-trap based *Dot1L* KO mouse line was established in our laboratory. In this mouse, we also were able to observe the reported lethality at approximately the same time during development. In addition, we observed a severe anemia in the *Dot1L* KO embryos. We performed colony forming unit (CFU) assays on yolk sac cells at around the time of the observed anemia and lethality (E10.5). These analyses confirmed that, indeed, there was severely deficient hematopoiesis (specifically, erythropoiesis) in the *Dot1L*-null yolk sac progenitors at this time<sup>7</sup>. In terms of mechanism, *Dot1L* deficiency blocked cell cycle progression and promoted apoptosis of these hematopoietic progenitor cells (Chapter 3). To investigate

the molecular mechanism, regulation of the expression of genes that are critical for hematopoiesis by DOT1L was analyzed. We initially observed that *Gata-2* expression appeared to be regulated by DOT1L. As a result of further analysis, we concluded that a simple reduction of *Gata-2* in the progenitors would not lead to the hematopoietic defects that we described. We then focused on the failure of the progenitors to thrive, and/or proliferation in response to EPO stimulation. Gene expression analysis revealed that *Trpc6*, a channel that plays a critical role in EPO mediated calcium influx, was significantly down regulated in *Dot1L* KO progenitors. Associated with this down-regulation was the observation of an abnormal intracellular calcium response in *Dot1L* KO progenitors after EPO treatment (Chapter 4). Taken together, the data support our conclusion that abnormal EPO responses in erythroid progenitor cells lead to excessive increases in calcium flux. This excessive increase leads to a failure of the progenitor cells to thrive and proliferate, and ultimately induces the cells to initiate an apoptosis program. The induced death of these cells is consistent with all of our cellular and molecular data, including the defective cell cycle progression and increased apoptosis of the erythroid progenitors, the reduced colony formation, and the embryonic anemia. The data help to broaden our understanding of the cellular and biological functions of *Dot1L*. Furthermore, this in depth molecular analysis provides us more insight into the intricate regulation of embryonic hematopoiesis.

## 5.1 The diverse functions of DOT1

The unique properties of DOT1 are reflected in two aspects. First, unlike most histone lysine methyltransferases, it does not contain the conserved SET domain, which is required for enzymatic activity and has been considered a signature structure for this

class of enzymes. Instead, its catalytic domain contains conserved sequence motifs similar to class I methyltransferases such as DNA methyltransferase (DNMT) and the protein arginine methyltransferase PRMT1<sup>89,127</sup>. Second, its substrate, H3K79 is located at the loop1 region within the globular domain of histone H3, which is quite different from most other important histone marks on tails of histones H3 or H4<sup>91</sup>. In addition, DOT1 in yeast and its homolog in other species is the only enzyme responsible for H3K79 methylation<sup>7,12</sup>. Therefore its function is considered non-redundant and indispensable for biological process.

*Dot1* was first identified in a genetic screen for genes whose overexpression disrupts telomeric silencing<sup>97</sup>. Interestingly, both overexpression and deletion, as well as mutation on H3K79 destroy silencing at telomeres<sup>10</sup>. It has been proposed that the balance between Sir proteins and the level of H3K79 methylation is important for regulating heterochromatin formation at telomeres, since the recruitment and binding of Sir proteins are critical for the establishment of telomere and telomere-proximal DNA silencing<sup>10</sup>. It needs to be noted that recent studies have challenged this established model and implied that DOT1 may not as critical in heterochromatin formation as previously thought<sup>146,147</sup>.

Besides its function in telomere silencing, *Dot1* and H3K79 methylation are also involved in transcriptional regulation. In yeast, 90% of the genome is enriched with H3K79 methylation and is actively transcribed at the same time. Meanwhile, the other 10% of the genome lacks H3K79 methylation, but is enriched with Sir proteins<sup>10,103</sup>. In *Drosophila*, chromatin immunoprecipitation coupled with gene expression microarray (ChIP-chip) revealed a positive correlation between H3K79 di-methylation and active

gene transcription<sup>105</sup>. Using a mammalian cell line, it was shown that H3K79 methylation marks are localized within the body of actively transcribed genes, and H3K79 methylation levels correspond to transcriptional activity<sup>13</sup>. Data from this dissertation are generally consistent with this view. In G1E cells, H3K79 di- and tri-methylation were found enriched in important regulatory elements of the actively transcribed *Gata-2* locus. In the silenced *pu.1* locus, no H3K79 di- and tri-methylation enrichment was observed at the promoter region, nor in the upstream regulatory elements<sup>7</sup>. In the *Trpc6* locus of bone marrow cells, where it is actively expressed, H3K79 di- and tri-methylation were much higher than in the *Trpc6* locus in liver cells expressing very low levels of *Trpc6*.

Studies using mammalian cell lines and ES cells revealed an interesting phenomenon that the loss of *Dot1L* results in transcriptional changes in only a subset of genes, despite the fact that H3K79 methylation is a universal modification<sup>13,108</sup>. My results are consistent with these observations. Among more than twenty genes analyzed, only a few of them are affected by *Dot1L* deficiency<sup>7</sup>. All of these observations lead to a hypothesis that only a subset of developmentally regulated mammalian genes requires DOT1L for their normal expression. One possible explanation for this phenomenon is that DOT1 might be functionally antagonistic to epigenetic silencing. Certain mammalian gene-silencing complexes (e.g. HP1 or Polycomb proteins) and the repressive histone mark H3K9 are found to exist over proximal transcribed regions of certain genes. The presence of these silencing proteins constitutes the requirement of *Dot1L* function. The location of H3K79 on the top and bottom of surfaces of the nucleosome suggests that its methylation could change the interaction between the nucleosome and other proteins, including silencing proteins. H3K79 di- and tri-methylation enriched within proximal

transcribed regions of a gene locus may change the critical protein-protein interactions required for a repressive chromatin conformation, therefore enabling promoter escape by elongation complexes under such conditions<sup>13</sup>. Another possible explanation is derived from purification of a number of transcriptional elongation complexes. Since genome-wide localization studies implied a potential role for H3K79 methylation in transcription elongation, several elongation complexes have been analyzed to ascertain the involvement of DOT1L<sup>14,148</sup>. It turns out that DOT1L is indeed included in some complexes, but not in others. It is likely that multiple transcriptional elongation complexes may exist, and DOT1L only associates with a subset of these complexes. When *Dot1L* is absent, expression of only those genes whose transcription requires a DOT1L-containing elongation complex will be affected<sup>92</sup>. These are some possible explanations that have not been fully tested. Furthermore, another purified DOT1L-containing complex includes both transcription activators (ENL) and repressors (SIRT1)<sup>149</sup>. It seems that DOT1L could mediate both transcriptional activation and repression through facilitating and integrating the opposing forces of positive and negative factors. Therefore, the mechanism of transcription regulation by DOT1 still remains a puzzle. In most cases, it seems that DOT1 plays its role in a locus-specific manner.

DOT1L has been implicated in cell cycle progression in ES cells as well as primary erythroid progenitor cells. In ES cells, the proliferation and pluripotency were not affected upon *Dot1L* deficiency, but severe proliferation defects were observed during differentiation, resulting in G2/M phase accumulation<sup>108</sup>. In contrast to that observed in ES cells, *Dot1L* KO in erythroid progenitor cells in our experiments showed



an accumulation at the G0/G1 phase<sup>7</sup>. This suggests that the cell cycle effects of DOT1L might be cell type-specific, or ligand-selective. The reason for this specificity in cell cycle regulation by DOT1L deserves further studies in the future.

*Dot1L* plays an important role in embryonic development. A *Dot1L* KO mouse line established by another group showed lethality by E10.5 during organogenesis of the cardiovascular system<sup>12</sup>. Our lab generated a gene-trap based *Dot1L* KO mouse line. We also observed embryonic lethality by E13.5, with severe anemia in *Dot1L* KO embryos<sup>7</sup>. In spite of this discrepancy (potentially due to the different methods used to generate the different mutant embryos), both results indicate that DOT1L is absolutely required for mouse embryonic development.

*Dot1L* KO embryos demonstrate cardiovascular defects, indicating that it plays a significant role in development of the cardiovascular system<sup>12</sup>. Interestingly, this may not be the major reason for the embryonic lethality in these mice. A cardiac-specific *Dot1L* KO mouse line showed that *Dot1L* deficiency in cardiomyocytes does not cause embryonic lethality<sup>114</sup>. In our experiments, severe anemia was observed and confirmed in *Dot1L* KO embryos. Since defective hematopoietic development is a common cause of embryonic death during mid gestation, we estimate that the hematopoietic defect upon *Dot1L* deficiency would be the major contributor to embryonic lethality. Future studies with hematopoietic-specific *Dot1L* KO model are required to confirm our prediction.

Besides the important role in normal embryonic and adult hematopoiesis, transcriptional activation by DOT1L mediated H3K79 methylation is also exploited in leukemogenesis. Translocation of *MLL* gene in leukemia leads to the interaction between the translocation partners and DOT1L. Mistargeting of DOT1L to the gene targets of

MLL fusion proteins results in aberrant hypermethylation of H3K79 and constitutive transcriptional activation of these genes. This, in turn, is thought to result in leukemic transformation<sup>115,118,119</sup>. *Dot1L* has been proposed to be a target for leukemia treatment. Although we need to bear in mind that DOT1L plays a role in normal hematopoiesis, it does not exclude the possibility of using small molecule inhibitors that target DOT1L, since normal hematopoietic stem/progenitors and leukemic cells may have different sensitivity to these inhibitors<sup>92,150</sup>.

## 5.2 Mechanistic studies of *Dot1L* in hematopoiesis

After the hematopoietic defect in *Dot1L* KO progenitor cells was identified, I have been trying to determine the mechanism. Since transcriptional regulation of important factors in hematopoiesis plays a critical role in proliferation and differentiation of progenitor and precursor cells, and DOT1L has been shown to be a potential regulator of transcription<sup>13</sup>, I started the mechanistic study by analyzing the transcription pattern changes of genes that are important in hematopoiesis.

The expression pattern changes in *Gata-2* and *pu.1* in *Dot1L* KO progenitors drew our attention at first. There is a 50% reduction of *Gata-2* and several fold increase of *pu.1* at the transcriptional level in c-kit<sup>+</sup> yolk sac cells when *Dot1L* is knocked out. GATA-2, GATA-1, and PU.1 are lineage-specific transcription factors expressed in multipotential progenitors<sup>5</sup>. Activation of specific transcription factors facilitates lineage commitment of progenitor cells down a particular differentiation pathway. GATA family proteins are essential for erythroid lineage development, while PU.1 is required for development of the myeloid lineage. The antagonistic relationship between GATA family proteins and PU.1 was elucidated many years ago<sup>53</sup>. A facet of that regulation is at the stem cell or

multipotential progenitor stage, where GATA-2 is expressed and inhibits PU.1 function by preventing its binding with its coactivator C-Jun. When progenitor cells develop along the erythroid pathway, GATA-2 is replaced by GATA-1, and PU.1 function is still inhibited by GATA-1. When progenitor cells develop into the myeloid lineage, both GATA-2 and GATA-1 are repressed and PU.1's function becomes dominant<sup>38,49,54,151</sup>. The *Gata-2/pu.1* expression pattern in Dot1L KO progenitors is in accord with the erythropoietic defect observed in CFU assay<sup>7</sup>. Therefore, my first hypothesis proposed that *Dot1L* mutation results in transcription pattern change of *Gata-2* and *pu.1*, which in turn causes specific erythroid development defect. However, it was not fully supported by subsequent experiments. First, only *Gata-2*'s expression is directly regulated by DOT1L. The expression of *pu.1* is not affected by *Dot1L* deficiency, as shown in K562 cells. Second, the reduction of *Gata-2* itself will not cause observable hematopoietic defect, at least in embryonic stage. It is possible that the increase of *pu.1* expression observed in yolk sac cells is a reflection of accumulated developmental defect, rather than the direct transcription regulation by DOT1L. The c-kit<sup>+</sup> progenitor cells are not a homogeneous population. They consist of multipotential progenitors as well as lineage committed progenitors such as common myeloid progenitors, granulocyte-monocyte progenitors and megakaryocyte-erythrocyte progenitors<sup>152</sup>. The multipotential progenitors "prime" several different lineage-specific program of gene activity (e.g. transcription factors, cytokine receptors) at relatively low level. Once the multipotential progenitors are committed to one particular lineage, upregulation of genes associated with the appropriate lineage and downregulation of genes with other lineages occur<sup>49,153</sup>. We speculate that the altered expression patterns of GATA2 and PU.1 are the *result*, rather

than the *cause* of the defective progenitor function. Increased apoptosis of cells responding to EPO would result in a pool of progenitors that lack erythroid and are enriched in a myeloid-generating population. These myeloid progenitor cells would have increased PU.1 expression and decreased GATA2, as has been reported<sup>50,52</sup>.

An important reason for the specific erythropoietic defect is the inappropriate response to erythroid growth stimuli. EPO is considered the master regulator of erythropoiesis and one of its important functions is to induce calcium influx in the progenitor cells through TRPC proteins<sup>4,135</sup>. In mouse, only *Trpc2* and *Trpc6* are expressed in the erythropoietic lineage. The stoichiometry of expression of *Trpc2* and *Trpc6* is an important mechanism for hematopoietic cells to regulate calcium influx in response to EPO stimulation. The ratio of TRPC2/TRPC6 expression on the cell membrane is thought to be required to regulate a normal EPO response. Changes in the expression of either of these two factors will affect this ratio and result in abnormal calcium influx and potentially, in defective hematopoiesis<sup>74,84,85,135</sup>. Gene expression analysis in progenitor cells revealed a significant reduction of *Trpc6* in the absence of *Dot1L*. Thus, my second hypothesis was that *Dot1L* deficiency results in down regulation of *Trpc6*, and leads to a change in the stoichiometric ratio of TRPC6:TRPC2, which in turn, results in abnormal calcium influx in response to EPO during erythropoiesis. Generally, this hypothesis has been supported by the data. The expression of *Trpc6* is closely correlated with H3K79 methylation status in the locus. In *Dot1L* KO yolk sac cells, *Trpc6* expression is dramatically decreased at both the mRNA and protein levels. In bone marrow cells, where *Trpc6* is highly expressed, the *Trpc6* locus is enriched with H3K79 di- and tri-methylation. In liver cells, where *Trpc6* is silenced, the H3K79

methylation level is much lower. This strong correlation implies that DOT1L or H3K79 methylation may play a role in the regulation of *Trpc6* expression. Moreover, a calcium imaging assay showed that EPO treatment elicits a significantly increased calcium influx in *Dot1L* KO yolk sac cells. It would have been nice if the erythropoietic defect in *Dot1L* KO could be rescued by EGTA treatment. We speculate that this was due to the abrupt chelating effect of EGTA rendered by its specific chemical properties, resulting in quantal changes in Calcium chelation<sup>142</sup>. Other modes of Calcium chelation could be tried in future experiments.

### 5.3 Future directions

*Dot1L* has been shown to play a critical role in both embryonic and adult hematopoiesis by our group and others. But the molecular mechanism(s) of *Dot1L* function during normal hematopoiesis has not been fully investigated. Using a conditional *Dot1L* KO model, two other groups demonstrated *Dot1L*'s role in maintaining normal adult hematopoiesis<sup>15,115</sup>. Unfortunately, they did not carry out any mechanistic studies or propose a hypothesis about *Dot1L* function in hematopoiesis. In my study, two hypotheses have been proposed and one of them is largely supported by the data. However, to fully prove the hypothesis and more thoroughly investigate *Dot1L* function in hematopoiesis, more extensive experiments need to be done. In the following part, some of the important directions will be discussed.

In my hypothesis, *Trpc6* is the major target gene affected by *Dot1L* KO. Down regulation of *Trpc6* is considered the major cause of abnormal calcium influx and defect in erythropoiesis. It is necessary to perform a *Trpc6* overexpression experiment in *Dot1L* deficient progenitor cells. If *Trpc6* is indeed critical in this process, restoration of *Trpc6*

to relatively normal levels in *Dot1L* deficient progenitor cells should rescue the erythropoietic defect, at least to some extent. In this experiment, analysis of c-kit<sup>+</sup> yolk sac cells would not be the ideal system due to the limitations in cell numbers in each yolk sac. In addition, it has been recognized that the nature of yolk sac progenitor is transient, and thus, not convenient to do genetic manipulation<sup>154</sup>. In contrast, ES cell differentiation approaches are very useful in studying erythropoiesis. Established protocols to induce ES cell into the hematopoietic lineages are available. Hematopoietic differentiation from ES cells proceeds in a synchronous manner, which facilitates cellular and molecular studies. Genetically modified ES cells can be used to study the effects of the genetic modification on hematopoietic differentiation<sup>4</sup>. This would be especially helpful in our studies, since *Dot1L* KO is embryonically lethal. Our laboratory is making a conditional *Dot1L* KO mouse line. In this mouse line, an important exon in *Dot1L* locus, required for methyltransferase activity<sup>12</sup>, is flanked by LoxP. The mouse also expresses Cre-estrogen receptor, which will produce Cre recombinase upon Tamoxifen. When the cells from the mouse line are treated with Tamoxifen, *Dot1L* will be functionally deleted. Embryonic fibroblast cells (MEF) can be easily derived from embryos from this mouse line to produce induced pluripotent stem cells (iPS cells). With the iPS cells, we can do many kinds of analyses, including induced knockout of *Dot1L*, hematopoietic differentiation of *Dot1L* conditional KO iPS cells, gene expression studies, as well as overexpression of *Trpc6* in *Dot1L* KO cells. By doing these experiments, the function of *Trpc6* in the regulation of hematopoiesis by *Dot1L* will be confirmed, and my hypothesis will be further supported.

As discussed earlier, the transcriptional activation of DOT1L-mediated H3K79 methylation also plays an important role in leukemogenesis. Although we need to fully understand DOT1L's function in normal hematopoiesis, it does not rule out the feasibility of using DOT1L as a target in leukemia treatment. Recently, several groups have reported the development of potent DOT1L-specific inhibitors<sup>150,155</sup>. Some of the inhibitors show highly selective inhibition of DOT1L methyltransferase activity, and exhibit only weak or no activities against other histone lysine or arginine methyltransferases. The high selectivity was confirmed by X-ray crystal structure of a DOT1L-inhibitor complex<sup>155</sup>. It is possible that normal hematopoietic stem cells/progenitor cells and leukemic cells may have different sensitivities to DOT1L inhibitors. Therefore in the future, it would be necessary to compare the sensitivity of normal hematopoietic stem/progenitor cells with leukemic cells to various potent DOT1L inhibitors. By doing this, we can determine which type of inhibitor has more selectivity against leukemic cells and what dosage of the inhibitors is specifically inhibitory in leukemic cells, but is tolerable in normal cells.

One more concept that we need to keep in mind is that H3K79 methylation is a universal histone mark. Accordingly, *Dot1L* expression is ubiquitous at different stages of embryonic development, from as early as E7.5 to E12.5<sup>12</sup>. It is obvious that *Dot1L* plays its role in various aspects of development. Even if my second hypothesis about calcium influx regulation by *Dot1L* shows promise, we need to keep our mind open to other possibilities. For instance, one important EPO signaling pathway is mediated by JAK2 and STAT5. One of the crucial target genes of STAT5 is the anti-apoptotic molecule Bcl-X<sub>L</sub><sup>6</sup>. In my studies, I observed more progenitor cells undergoing apoptosis

during erythropoiesis when *Dot1L* is deficient. A likely explanation, as I discuss previously, is due to abnormally high levels of intracellular calcium in *Dot1L* KO progenitor cells. Another possibility would be dysregulation of factors in the apoptotic pathway, such as Bcl-X<sub>L</sub>. In the future, it would be helpful if we can analyze and compare the expression and regulation of critical apoptotic factors between *Dot1L* KO progenitors and wild type cells during erythropoiesis. In addition, I observed cell cycle defect in *Dot1L* KO progenitor cells during erythropoiesis. Our results and others also point out that the cell cycle effect of *Dot1L* might be tissue- and cell type-specific<sup>7,92,108</sup>. It would be interesting to perform cell cycle analysis in the erythropoietic lineage and other cell types in *Dot1L* KO embryos. If the defect is specific and limited to erythropoietic cells, this would be another possible mechanism for the erythropoietic defect.

#### 5.4 Conclusion remarks

In this dissertation, I demonstrated the role of DOT1L methyltransferase in embryonic development and erythropoiesis, and in the regulation of critical genes in hematopoiesis. The morphological, cell cycle, and apoptotic defects in erythropoiesis suggest *Dot1L* KO progenitors fail to respond to lineage specific growth factor(s). This has been supported by my hypothesis indicating that EPO signaling pathway is interfered with in *Dot1L* KO progenitors. Future work using powerful *in vitro* models, such as iPS cells, will provide more consolidated support for my hypothesis and further elucidate the pathway. Exploration of other possible mechanisms will give us a more comprehensive understanding of DOT1L's role in hematopoiesis, which will not only provide insight into the complex regulatory network of normal hematopoiesis, but also shed light on the potential therapeutic aspects of a variety of clinical diseases.



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## Appendix

### Curriculum Vitae

YI FENG

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#### Education

- 1992-1997 Bachelor of Medicine, Tianjin Medical University, Tianjin, P. R. China
- 2005-2007 PhD program in Biology, Cleveland State University, OH, USA
- 2007-now PhD program in Department of Pathology, Kansas University Medical Center, KS, USA

#### Research Experience

- Collaboration in the research of the dentinogenesis imperfecta type II locus. (*Honored the second prize of 2002 National Scientific Research*)

Summary: active involvements in lab research, clinical treatment for the patients, samples and information collection, and translation of English papers to Chinese versions, 1999-2002

- Rotation in Dr. Boerner's lab in Cleveland State University on *S. cerevisiae* genetics, 05/2006-09/2006

Summary: preliminary work on a large scale genomic screen of genes that play critical roles in meiotic pathway, including amplification of genomic library of *Saccharomyces cerevisiae*, transformation of yeasts with the library, optimization of research condition.

- Rotation in Dr. Li's lab in Cleveland State University on Telomere structure and function study, 10/2006-04/2007

Summary: study on the function and interaction of critical telomere protein factors using *T. Brucei* as model system.

- Work in Dr. Fields' lab in KU Medical Center, 07/2007-now

Summary: work on the project on the function of Dot1L histone methyltransferase in early embryonic development using mouse model. Dot1L is the only enzyme for methylation of H3K79 and its function is non-redundant. We found that Dot1L is absolutely required for mouse embryonic development. Dot1L knock-out embryos undergo severe anemia. The anemia defect was confirmed by Colony Forming Units Assay using both primitive and definitive hematopoietic progenitor cells derived from yolk sacs. Interestingly, we found that Dot1L knock-out results in specific defect in erythroid lineage, but not in myeloid lineage. Also, during erythropoiesis, more Dot1L knock-out cells undergo cell-cycle defect and apoptosis. Gene expression analysis showed that among a set of genes important in hematopoietic development, only a few of them were affected by Dot1L deficiency. TRPC2 and TRPC6 are voltage-independent calcium channels and they form a protein complex on hematopoietic lineage cell surface to regulate calcium influx when the cell is exposed to Epo. We found a reduction of trpc6 expression level in Dot1L knock-out progenitor cells, while trpc2 level remained unaffected. Our current hypothesis is that Dot1L mutant results in decreased trpc6 expression, which in turn leads to abnormal calcium influx in response to Epo. This abnormal calcium level results in erythropoietic defect.

### **Working experience**

1997 fall--2005 spring, working in Stomatological Hospital of Tianjin Medical University. Major work included diagnosis of dental diseases, preventive treatment of dental and periodontal disorders, dental caries removal and cavity fillings, cosmetic dentistry (including whitening teeth, front teeth restoration by light activated resin, etc), root canal treatment, root end surgery (apicoectomy).

### **Techniques familiar with**

**Techniques in molecular and cellular biology:** Mouse Colony Maintenance; Mouse Genotyping (using tissue from ear or tail); DNA manipulation including isolation of genomic DNA, Polymerase Chain Reaction (PCR); RNA manipulation including RNA isolation, Reverse Transcriptase PCR, Real-Time PCR for gene expression analysis; Mouse yolk sac dissection and isolation of hematopoietic progenitor cells; Histone extraction from mouse embryonic fibroblasts; Colony Forming Units Assay for hematopoietic potential study; Giemsa Staining for blood smear; Cell-cycle Analysis; Apoptotic Analysis; Regular Cell Culture technique; Lentiviral Vector Transfection and Transduction to knockdown gene expression; Bone Marrow Cell extraction from mouse; Western Blot Analysis; Chromatin Immunoprecipitation (ChIP) Assay; Flow Cytometry Analysis and fluorescence activated cell sorting; Hematopoietic Stem Cell maintenance and differentiation.

**Techniques from Medical School:** Dental cavity preparation, dental cavity filling, aseptic technique, alveolar bone surgery, teeth stabilization after traumatic dental injury, root end surgery.

### **Publication**

- Ligeng Wu, Zhiyan Hou, **Yi Feng**. Malignant Melanoma on the Oral Mucosa. *Journal of Modern Stomatology*. 1998, 12:19
- Jun Zhao, **Yi Feng**. The finding of a family of the Dentinogenesis Imperfecta Type II. *Chinese Journal of Medical Genetics*. 1999, 16(1):43
- **Yi Feng**, Ruoyu Yang. The Application of Polymolecular Material on Removable Dentures. *Journal of Tianjin Medical University*. 2002, 8(2):250
- **Feng Y**, Yang Y, Ortega MM, Copeland JN, Zhang M, Jacob JB, Fields TA, Vivian JL, Fields PE.. Early mammalian erythropoiesis requires the Dot1L methyltransferase. *Blood*. 2010 Nov 25;116(22):4483-91.
- Copeland JN, **Feng Y**, Neradugomma NK, Fields PE, Vivian JL. Notch signaling regulates remodeling and vessel diameter in the extraembryonic yolk sac. *BMC Dev Biol*. 2011 Feb 25; 11:12.